Maintenance of female colour polymorphism in the coenagrionid damselfly *Coenagrion puella*.

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1. Referent: Prof. Dr. Georg Rüppell

2. Referent: Dr. Jens Rolff

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Abstract

How colour polymorphisms are maintained is still an unresolved question. Selection should favour the morph best adapted (Moran 1992). Furthermore, the maintenance of a polymorphic system is supposed to be costly, therefore it seems only profitable under quickly or steadily changing environmental conditions (Moran 1992).

Colour polymorphism is a common trait in damselflies, especially in female coenagrionids (Odonata: Zygoptera). This has been discussed in literature for more than 100 years and several hypotheses to explain these poylmorphisms have been developed (Fig. 1).

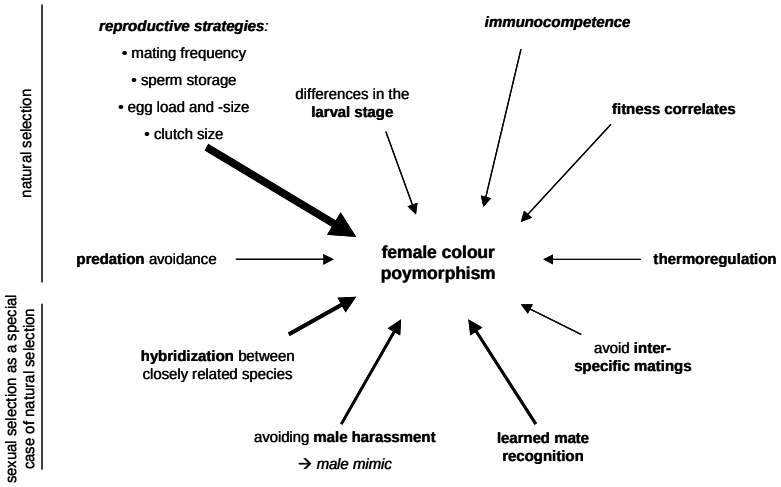

Figure 1: Factors that might have an impact on the maintenance of this colour polymorphism. Width of an arrow presents the assumed influence of the factor. Factors in itallics are dealed with in this thesis.

As a model organism I chose the azur damselfly, *Coenagrion puella*. In this species males are blue while females show three colour morphs, green, blue and intermediate. The question is how these female colour morphs are maintained. The focus of the presented work to answer this question is on differences in blackcontent

and colouration on thorax and abdomen of male and polymorphic female *C. puella*, furthermore on differences in immune parameters and reproductive strategies.

For black content no differences between the female morphs were found. Males however have a smaller black content than females. In colour composition it was found that blue females are of a different blue than males, and all three female morphs differ in colour composition. The haemolyph's haemocyte numbers and Phenoloxidase activity (PO) and their regulation under the risk of predation and parasitism in the larval stage were investigated as immune parameters. Here differences between the sexes were found. This led to the question, whether there are similar differences between the female morphs. Therefore haemocyte numbers and PO in adult male and polymorphic females were investigated. Furthermore differences in mortality in the presence of a newly introduced entomopathogenic fungi and parasite numbers in the field were examined. For all this parameters no differences between the female morphs were found but differences between the sexes. For reproductive strategies it is discussed, which impact the between the morphs differing egg shapes could have on the choice of oviposition substrate.

From these results the question, how this polymorphism evolved and if it evolved parllel in all coenagrionid species, arose. To answer this a new molecular phylogenetic tree of the coenagrionids was build. So far it seems that the female colour polymorphism evolved several times within this group.

In summary, I conclude that none of the in figure 1 presented factors maintaines this polymorphism alone, but rather a combination of all of them. If I included, that the polymorphism might have evolved several times within the coenagrionids under differing selection pressures, the question of the maintaining factors becomes even more complex.

Zusammenfassung

Wie Farbpolymorphismen aufrecht erhalten werden ist immer noch eine ungeklärte Frage, insbesondere da durch Selektion immer die best-angepasste Morphe bevorteilt werden sollte (Moran 1992). Gleichzeitig sollte die Aufrechterhaltung eines polymorphen Systems mit zusätzlichen Kosten verbunden sein, so dass dies nur unter sich schnell oder stetig ändernden Umweltbedingungen rentabel erscheint (Moran 1992).

Farb-Polymorphismus ist bei Kleinlibellen ein weit verbreitetes Merkmal innerhalb der Weibchen vieler Coenagrioniden-Arten (Odonata: Zygoptera). Dies wird in der Literatur schon seit über 100 Jahren diskutiert und verschiedenste Hypothesen zur Erklärung dieses Polymorphismus wurden entwickelt (Abbildung 1).

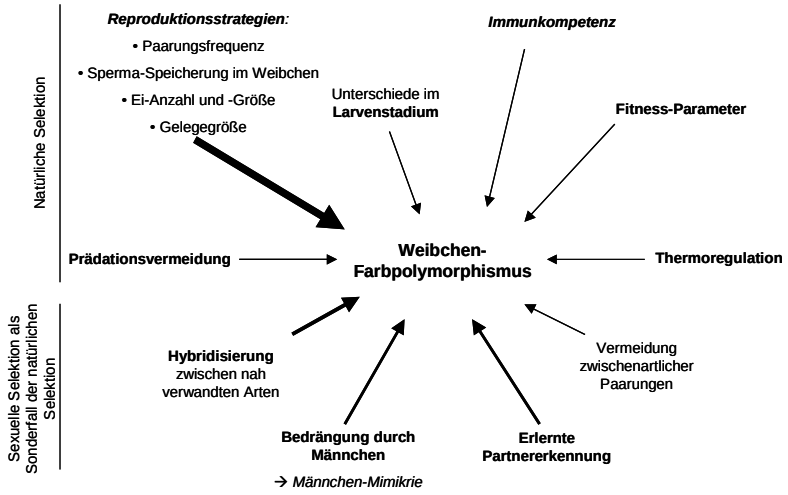

Abbildung 1: Diskutierte Faktoren, die für die Aufrechterhaltung des Farb-Polymorphismus eine Rolle spielen können. Die Stärke der Pfeile gibt den vermuteten Einfluss des Faktors wieder, in kursiv dargestellte Faktoren werden in der vorliegenden Arbeit behandelt.

Als Modellorganismus wurde hier die Hufeisenazurjungfer, *Coenagrion puella* gewählt. Bei dieser Art sind die Männchen blau, während es drei Weibchen-Farbmorphen gibt, grün, blau und intermediär. Dabei stellt sich die Frage, was diesen

Polymorphismus aufrecht erhält. In der vorliegenden Arbeit liegt der Schwerpunkt zur Beantwortung dieser Frage auf Schwarzanteil- und Farbunterschieden an Thorax und Abdomen der Männchen und Weibchenmorphen von *C. puella*, Unterschieden in Immunparametern und Reproduktion.

Für den Schwarzanteil konnte gezeigt werden, dass es keine Unterschiede zwischen den Weibchenmorphen gibt, die Männchen aber einen wesentlich geringeren Schwarzanteil haben. Für die Farbzusammensetzung wurde gefunden, dass blaue Weibchen ein anderes Blau als die Männchen haben und sich alle drei Weibchen-morphen in ihrer Farbzusammensetzung unterscheiden. Als Immunparameter wurden zunächst Haemocytengehalt und Phenoloxidaseaktivität (PO) der Haemolymphe und deren Regulation in Gegenwart von Prädatoren und Parasiten im Larvenstadium untersucht. Hier wurden auffällige Geschlechtsunterschiede gefunden, die zu der Frage überleiten, ob es auch zwischen den Weibchenmorphen solch auffällige Unterschiede in der Immunkompetenz gibt. Dazu wurden wiederum Haemocytengehalt und PO betrachtet, diesmal für adulte Männchen und alle drei Weibchenmorphen. Des weiteren wurden Mortalitätsunterschiede in Gegenwart eines neu eingeführten entomopathogenen Pilzes und Parasitenbefall im Freiland aufgenommen. Bezüglich dieser Parameter wurden keine Unterschiede zwischen den Weibchenmorphen von *C. puella* festgestellt, wohl aber große Unterschiede zwischen den Geschlechtern. Als unterschiedliche Reproduktionsstrategien wird diskutiert, wie sich die zwischen den Morphen verschiedenen Eiformen bezüglich der Eiablage-Substratwahl auswirken können.

Aus den dargestellten Ergebnissen entwickelte sich die Frage, wie dieser Polymorphismus evolutiv entstanden ist und ob er für alle Coenagrioniden parallel entstand. Um die Evolution des Farbpolymorphismus der Coenagrioniden zu

betrachten, wurde eine neue Phylogenie der Coenagrioniden erstellt und die Ergebnisse deuten darauf hin, dass der Weibchen-Farbpolymorphismus mehrmals innerhalb dieser Gruppe entstanden ist.

Abschließend kann ich feststellen, dass wohl keiner der in Abbildung 1 angesprochenen Faktoren alleine diesen Farbpolymorphismus aufrecht erhält, sondern vielmehr ein Zusammenspiel der verschiedensten Faktoren. Berücksichtigt man dabei, dass eventuell bei den verschiedenen Arten unterschiedliche Selektionsdrücke zur Entstehung des Polymorphismus beigetragen haben, so wird die Thematik noch komplexer.

‚... and the ordinary coloration of the two sexes is reversed, as we have just seen, in one species of Agrion.‘

(Darwin 1871)

‘Darwin clearly distinguished sexual from natural selection and regarded the two as often acting in opposition, sexual selection favouring traits such as bright coloration, ...’

(Fairbairn and Reeve 2001)

Maintenance of female colour polymorphism in the coenagrionid damselfly *Coenagrion puella*.

Introduction and Discussion

Colour polymorphisms are widespread within and among populations of several species of invertebrates (e.g. Vane-Wright 1975: Butterflies) and vertebrates (e.g. Wente and Phillips 2003: Pacific Tree Frog). Polymorphisms can be maintained by different selection pressures such as predation (Munday et al. 2003: Coral Reef Fish), camouflage (e.g. Majerus 1989: Peppered Moth) or mimicry (Begon et al. 1998). Often polymorphisms are restricted to one sex, as in yellowhammers *Emberiza citrinella* (Sundberg 1995) or guppies *Poecilia reticulata* (Gamble et al. 2003). In this case the colour morphs may be either totally independent of each other in terms of pattern and/or colour (e.g. Stuart-Fox et al. 2003) or one morph of the polymorphic gender may resemble the monomorphic gender, which has been

dubbed intra-specific mimicry (e.g. Andolfatto et al. 2003). This idea of one sex mimicing the other sex was already suggested by Darwin (1871) using one of the best described and widely discussed examples in insects: the damselflies.

Female polymorphism is common in coenagrionid damselflies (e.g. Johnson 1964: *Ischnura damula*; Robertson 1985: *I. ramburi*; Hinnekint 1987: *I. elegans*; Cordero 1990: *I. graellsii*; Andres & Cordero Rivera 2001: *Ceriagrion tenellum*). A few other families (e.g. Calopterygidae) exhibit male polymorphism (e.g. Tsubaki et al. 1997: *Mnais pruinosa costalis*), which is usually a polymorphism of wing patterning. In females, the term colour polymorphism is used for differences in black pattering (Johnson 1964 and 1966), in the colour itself (Robertson 1985) or a combination of both on thorax and abdomen. In general, it is purported that one of the female morphs resembles the male and therefore it is termed ‘andromorphic’ or ‘androchrome’, while the other female morphs are termed as ‘heteromorphic’ or ‘gynochrome’ (Johnson 1964, Cordero 1990). Johnson (1964; 1966) showed for *I. damula* and *I. Demerosa*, Cordero (1990) and Andres & Cordero (1999) for *I. graellsii* and *C. tenellum* and Sanchez-Guillen et al. (2005) for *I. elegans* that these colour polymorphisms are genetically determined, and this is most likely the case for all coenagrionid species.

Ever since Darwin discussed polymorphic damselflies with MacLachlan (Darwin 1871), several hypotheses have been suggested to explain the maintenance of this female colour polymorphism (table 1). Most of these theories are based on the idea that females are mimicking males (table 1, sexual selection). Even different approaches such as ‘learned mate recognition’ (Fincke 1994, Forbes 1994; Miller & Fincke 1999) still use the terms ‘andro’- and ‘gynomorph/-chrome’ and thereby imply that one female morph is male-like. Furthermore, often only the differences in the male’s response to the female morphs (models or tethered animals) are tested rather

than the differences in male responses towards males, based on the assumption that ‘androchrome’ females mimic males (e.g. Gorb 1998).

Some of the explanations for female colour polymorphism may be confounded because they do not consider the differences between human and insect vision. This is important when realizing that the ‘male mimic’ theory is based on the idea of an ‘andromorph’ female, as distinguished by human eye. Sherratt & Forbes (2001) discussed this problem and concluded that odonates probably have a different vision than we do, but might have a restricted colour vision compared to humans. However, in most species the resemblance is restricted to the colour, in coenagrionids usually blue and sometimes red, but there is no resemblance in the prominent black patterns. Another problem with the explanations revolving around male mimicking is that they do not provide an explanation for species with several ‘gynochrome’ female morphs, as in *I. elegans*. In this thesis I try to identify mechanisms by which the female colour polymorphism in *Coenagrion puella* may be maintained, using different approaches, from colour analyses over gender differences, immunocompetence and reproductive strategies to a new coenagrionid phylogeny, as summarised in table 1 and in the following outline.

Table 1: Hypotheses that explain the evolution and maintenance of colour polymorphisms in damselflies in the literature and which aspects are investigated in this thesis. All hypothesis are sorted according to whether they assume sexual or natural selection as basis for the maintenance of female colour polymorphism in coenagrionid damselflies. Roman numerals refer to the chapter in this thesis.

	Which selectional pressures might maintain the female colour polymorphism?	
	sexual selection	**natural selection**
In literature	• avoid harassemnt by mimicing males (Johnson 1964, Robertson 1985, Hinnekint 1987) • learned mate recognition (Miller and Fincke 1999, Fincke 2004) • morphs have different sexual interaction with different costs (Sirot and Brockmann 2001) • sex-related aposematism (Sherratt and Forbes 2001)	• sun protection (Ris 1906) • thermoregulation (Conrad and Pritchard 1988) • differences in larval nutrition? (Cordero 1992) • habituation (van Gossum et al. 1999) • frequency dependent selection (Andres et al. 2002) • natural cycles (Svensson et al. 2005)
this thesis	I. are blue females realy similar to the males in *C. puella*? ⟶ challenging male mimic	II. gender differ in immune reaction towards predators and parasites III. do the female morphs show different immune response in general or to parasites? IV. do the female morphs follow different reproductive strategies to optimise fitness?
	If blue females obviously are not male like and no further differences to the other female morphs can be found, can we assume at all, that this polymorphism is ancestral for all coenagrionid damselflies and therefore evolved for the same reasons ?	
	V. evolution of female colour polymorphism in coenagrionid damselflies with a new molecular phylogeny	

Thesis outline

Chapter I I started by analyzing the differences of black patterning and colouration between sexes and within the female morphs. The whole idea of females mimicking males is based on the human impression of the female morphs only. In *Coenagrion puella* the blue female morph differs obviously in black patterning from the male, which is supported by analyses of black area in this study. Therefore I examined, how similar the blue female really is to the male. In an R(ed)G(reen)B(lue) analysis of colour it was found, that the blue females are of a different blue than the males. Combining this with the better colour vision of insects and the non-existence of UV-signals in this species I propose that males should be able to distinguish blue

females from males, furthermore that males should recognize blue females as typical female from black patterning and colouration.

Conclusion If male mimic and hence sexual selection can be excluded for the maintenance of female colour polymorphism in *C. puella* the question is what else is selected on. It seems plausible that costly traits as the immune system or reproduction are the most likely.

Chapter II This chapter presents gender differences in immune response towards predation and parasitism in *C. puella*. Larvae were reared under the risk of predation and/or parasitism. General differences in haemocyte load and Phenoloxidase (PO) activity were found between the sexes as well as differences in the modification of the immune response under the risks presented. These results lead to the question, if there was anything like females mimicking males, are the ‘male like’ females also male like in immunocompetence or do they make a group of their own?

Chapter III If the idea of females mimicking males to avoid male harassment was to be true, this might be maintained by different costs for immunity, as mating has been shown to reduce the immune defence in insects (Siva-Jothy et al. 1998, Rolff and Siva-Jothy 2002). Here I address this question by analyzing different immune traits for males and the female morphs of *C. puella*: (i) It is already shown in chapter I that the sexes but not the female morphs differ in melanin (= black) content, showing that the sexes but not the female morphs differ in the cuticle, a physico-chemical barrier that constitutes the first line in insect immune defense. (ii) Another component of the innate immune system is the cellular immunity. Here I present haemocyte counts for male and female *C. puella* and again, blue females are typical female and all females

differ from the males. (iii) The same was found for PO activity, which is a humoral component of immunity.

Moreover, I examined how the immune response is adapted to risks in the wild. Therefore (iv) freshly emerged *C. puella* were presented to a newly introduced entomopathogenic fungi and their survival was recorded. It was found that the sexes differ in survival but not the female morphs. (v) For parasite loads in the wild infestation with water mites as an ectoparasite and gregarines as an endoparasite were investigated, but here no differences were found, neither between female morphs nor between sexes. This result indicates again, that blue females are typical female in *C. puella*.

Chapter IV As the female polymorphism seems not to be maintained by differences in immunocompetence, an other likely as also costly trait is reproduction (Wigglesworth 1959), especially as females optimize their fitness via this trait. Based on my hypothesis, that the female morphs follow different reproductive strategies clutch- and egg size as well as wing morphometry were analyzed. Females might differ in clutch- and/or egg-size, which might result in weight differences. This again might influence flight performance via wing loading (Rüppell 1989) or could be balanced by differences in wing morphometry.

Only differences in wing morphometry were found but none in clutch- or egg size. However, egg shape differs between the female morphs. This might indicate different preferences for the oviposition substrate chosen. Differences in wing morphometry could support this, if the female would need different flight maneuvering to reach the preferred substrate. It is discussed, how this could lead to varying mating preferences in males depending on the substrates present at a pond.

Conclusion From the physiological data I have obtained it seems to be clear that the blue female *C. puella* seem to be typical female. The only differences detected are in egg shape. Another question which arose quite early during my work on this thesis is the evolution of the female colour polymorphism in coenagrionid damselfly in general. While working on chapter I it became obvious, that there might be at least two different groups of polymorphism within the coenagrionids; (i) presented by *Coenagrion puella*, where the 'male like' females have a typical female black patterning and (ii) presented by *Ischnura elegans*, where all female morphs are pretty similar in black patterning to the males.

Chapter V The term male mimic implies that males were blue when the female colour polymorphism arose. But if males were green in the past, as Haldane's sieve (the recessive allele should be the ancestral one) suggests, it poses the question: "Which morph is the model?" (Sherratt 2001). In this chapter I present a new molecular phylogeny based on the mitochondrial gene COII for coenagrionid damselflies based on data from gene bank as well as own samples. With this phylogeny I try to answer, whether the female polymorphism arose once or several times within the Coenagrionidae and what evolutionary steps might have led to the assumed two groups of colour polymorphism.

References

Andolfatto, P., Scriber, J. M. & Charlesworth, B. 2003. No association between mitochindrial DNA haplotypes and a female-limited mimicry phenotype in *Papilio glaucus*. *Evolution*, **57,** 305-316.

Andres, J. A. & Cordero, A. 1999. The inheritance of female colour morphs in the damselfly *Ceriagrion tenellum* (Odonata, Coenagrionidae). *Heredity*, **82,** 328-335.

Andres, J. A. & Cordero Rivera, A. 2001. Survival rates in a natural population of the damselfly *Ceriagrin tenellum*: effects of sex and female phenotype. *Ecological Entomology*, **26,** 341-346.

Andres, J. A., Sanchez-Guillen, R. S. & Cordero Rivera, A. 2002. Evolution of female colour polymorphism in damselflies: testing the hypothesis. *Animal Behaviour*, **63,** 677-685.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. *Heredity*, **2,** 349-368.

Begon, M., Harper, J. & Townsend, C. 1998. *Oekologie*. Heidelberg: Spektrum Akademischer Verlag.

Conrad, K. F. & Pritchard, G. 1988. Female dimorphism and physiological colour change in the damselfly *Agria vivida* Hagen (Odonata: Coenagrionidae). *Canadian Journal of Zoology*, **67,** 298-304.

Corbet, P. S. 1999. *Dragonflies - behaviour and ecology of Odonata*. Colchester: Harley Books.

Cordero, A. 1990. The inheritance of female polymorphism in the damselfly *Ischnura graellsii* (Rambur) (Odonata: Coenagrionidae). *Heredity*, **64,** 341-346.

Cordero, A. 1992. Density-dependent mating success and colour polymorphism in females of the damselfly *Ischnura graellsii* (Odonata: Coenagrionidae). *Journal of Animal Ecology*, **61,** 769-780.

Darwin, C. 1871. *The Descent of man*. London: John Murray.

Fairbairn, D. J. & Reeve, J. P. 2001. Natural Selection. In: *Evolutionary Ecology. Concepts and case studies* (Ed. by Fox, C. W., Roff, D. A. & Fairbairn, D. j.), pp. 424. New York: Oxford University Press.

Fincke, O. M. 1994. On the difficulty of detecting density-dependent selection on polymorphic females of the damselfly Ischnura graellsii: failure to reject the null. *Evolutionary Ecology*, **8,** 328-329.

Fincke, O. M. 2004. Polymorphic signals of harassed odonates and the males that learn them support a novel frequency-dependent model. *Animal Behaviour*, **67,** 833-845.

Folstad, I. & Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. *The American Naturalist*, **139,** 603-622.

Forbes, M. 1994. Test of hypotheses for female-limited polymorphism in the damselfly, *Enallagma boreale* Selys. *Animal Behaviour*, **47,** 724-726.

Futuyma, D. 1990. *Evolutionsbiologie*. Basel, Boston, Berlin: Birkhaeuser Verlag.

Gamble, S., Lindholm, A. K., Endler, J. A. & Brooks, R. 2003. Envirnomental variation and the maintenance of polymorphism: the effect of ambient light spectrum on mating behaviour and sexual selection in guppies. *Ecology Letters*, **6,** 463-472.

Gorb, S. N. 1998. Visual cues in mate recognition ba males of the damselfly, *Coenagrion puella* (L.) (Odonata: Coenagrionidae). *Journal of Insect Behaviour*, **11,** 73-92.

Hamilton, W. D. & Zuk, M. 1982. Heritable true fitness and bright birds - a role for parasites. *Science*, **218,** 384-387.

Hinnekint, B., O, N. 1987. Population dynamics of Ischnura E. elegans (Vander Linden) (Insecta:Odonata) with special reference to morphological colour change, female polymorphism, multiannual cicles and their influence on behaviour. *Hydrobiologia*, **146,** 3-31.

Johnson, C. 1964. The inheritance of female dimorphism in the damselfly, *Ischnura damula*. *Genetics*, **49,** 513-519.

Johnson, C. 1966. Genetics of female dimorphism in *Ischnura demorsa*. *Heredity*, **21,** 453-459.

Majerus, M. E. N. 1989. Melanic polymorphism in the peppered moth, Biston-Betularia, and other Lepidoptera. *Journal of Biological Education*, **23,** 267-284.

Maynard Smith, J. & Harper, D. 2003. *Animal Signals*. New York: Oxford University Press.

Miller, M. N. & Fincke, O. M. 1999. Cues for mate recognition and the effect of prior experiences on mate recognition in *Enallagma* damselflies. *Journal of Insect Behaviour*, **12,** 801-814.

Moran, N. A. 1992. The evolutionary maintenance of alternative phenotypes. *The American Naturalist*, **139,** 971-989.

Munday, P. L., Eyre, P. J. & Jones, G. P. 2003. Ecological mechanisms for coexistence of colour polymorphism in a coral-reef fish: an experimental evaluation. *Oecologia*, **442,** 519-526.

Rehn, T. & Crews, D. 2001. Sex and Gender. In: *Evolutionary Ecology. Concepts and case studies* (Ed. by Fox, C. W., Roff, D. A. & Fairbairn, D. J.), pp. 424. New York: Oxford University Press.

Ris, F. 1906. Farbenvarietäten der Agrionide Nehalennia speciosa Charp. *Mitteilungen der schweizerischen entomologischen Gesellschaft*, **11,** 159-165.

Robertson, H. M. 1985. Female dimorphism and mating behaviour in a damselfly, Ischnuar ramburi: females mimicking males. *Animal Behaviour*, **33,** 805-809.

Rolff, J. & Siva-Jothy, M. T. 2002. Copulation corrupts immunity: A mechanism for a cost of mating in insects. *Proceedings of the National Academy of Sciences*.

Rüppell, G. 1989. Kinematic analysis of symmetrical flight manoeuvres of Odonata. *Journal of experimental Biology*, **144,** 13-42.

Sanchez-Guillen, R. A., Van Gossum, H. and Cordero Rivera, A. 2005. Hybridization and the inheritance of female colour polymorphism in two ischnurid damselflies (Odonata: Coenagrionidae). *Biological Journal of the Linnean Society*, **85**, 471-481.

Sherratt, T., N & Forbes, M., R. 2001. Sexual differences in coloration of Conagrionid damselflies (Odonata): a case of intraspecific aposematism? *Animal behaviour*, **62,** 653-660.

Sherratt, T. N. 2001. The evolution of female-limited polymorphisms in damselflies: a signal detection model. *Ecology letters*, **4,** 22-29.

Siva-Jothy, M., Tsubaki, Y. and Hooper, R. E. 1998. Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiological Entomology*, **23,** 274-277.

Sirot, L. K. & Brockmann, H. J. 2001. Costs of sexual interactions to females in Rammbur's forktail damselfly, *Ischnura ramburi* (Zygoteran: Coenagrionidae). *Animal Behaviour*, **61,** 415-424.

Stuart-Fox, D. M., Moussalli, A., Marshall, N. J. & Owens, I. P. F. 2003. Conspicouos males suffer higher predation risk: visual modelling and experimental evidence from lizards. *Animal Behaviour*, **66,** 541-550.

Sundberg, J. 1995. Parasites, plumage coloration and reproductive success in the yellowhammer, Emberiza citrinella. *OIKOS*, **74,** 331-339.

Svensson, E. I., Abbott, J. & Härdling, R. 2005. Female polymorphism, frequency dependence, and rapid evolutionary dynamics in naural populations. *The American Naturalist*, **165,** 000-000.

Tsubaki, Y., Hooper, R. E. & Siva-Jothy, M. T. 1997. Differences in adult and reproductive lifespan in two male forms of *Mnais pruniosa costalis* Selys (Odonata: Calopterygidae). *Researches on Population Ecology*, **39,** 149-155.

Van Gossum, H., Stoks, R., Matthhysen, E., Valck, F. & De Bruyn, L. 1999. Male choice for female colour morphs in *Ischnura elegans* (Odonata, Coenagrionidae): testing the hypotheses. *Animal Behaviour*, **57,** 1229-1232.

Vane-Wright, R. I. 1979. Towards a theory of the evolution of butterfly colour patterns under directional and disruptive selection. *Biological Journal of the Linnean Society*, **11,** 141-142.

Wente, W. H. & Phillips, J. B. 2003. Fixed green and brown color morphs and a novel color-changing morph of the pacific tree frog Hyla regilla. *The American Naturalist*, **162,** 461-473.

Wigglesworth, V. 1959. *Physiologie der Insekten*. Basel, Stuttgard: Birkhaeuser Verlag.

Chapter I

Signals - ‘any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective, because the receiver’s response has also evolved.’

(Maynard Smith and Harper 2003)

Gender and the eye of the beholder in coenagrionid damselflies

Abstract

The evolution and maintenance of colour polymorphism in female damselflies is unresolved: usually, one female morph resembles the blue male colour (andromorph) while one, or more, female morphs are seen as typically female (gynomorph). Damselfly species fall into distinct groups with respect to recent developments in mimicry theory: in some species females are perfect and in other species they are supposed to be imperfect mimics. However, the underlying assumption of one female morph looking male-like is mostly based on human vision. Therefore we investigated the black patterning and colour of the three female morphs in *Coenagrion puella*, an imperfect mimic, using image analysis as the most neutral tool available. In *C. puella* the blue female morph is seen as male-like, but we found that in terms of black patterning these females cannot be distinguished from the other female morphs, and are clearly different from males. Furthermore, the blue of andromorph females differs from the blue of males. This challenges the view that andromorph females are imperfect male mimics in this species. Intriguingly, however, the red content did not differ between blue males and females, and insects posses a specific red receptor.

Introduction

Colour polymorphisms are widespread within and among populations of several species (e.g. Vane-Wright, 1975; Svensson and Sinervo, 2000; Wente and Phillips, 2003) and are often restricted to one sex (e.g. Sundberg, 1995: Yellowhammer; Gamble *et al.,* 2003: Guppies). Often one morph of the polymorphic sex resembles the monomorphic sex, which has been dubbed intra-specific mimicry (e.g. Andolfatto *et al.,* 2003). This idea of one sex mimicking the other sex was already suggested by Darwin (1871) using one of the best described and widely discussed examples in insects: the damselflies.

Female polymorphism is common in coenagrionid damselflies (e.g. Johnson, 1964: *Ischnura damula*; Robertson, 1985: *I. ramburi*; Hinnekint, 1987: *I. elegans*; Cordero, 1990: *I. graellsii*; Andres and Cordero Rivera, 2001: *Ceriagrion tenellum*). In females, the term colour polymorphism is used for differences in black pattering (Johnson, 1964 and 1966), in the colour itself (Robertson, 1985) or a combination of both. In general, it is purported that one of the female morphs mimics the males and therefore it is termed ‘andromorphic’ or ‘androchrome’, while the other female morphs are termed as ‘heteromorphic’ or ‘gynochrome’ (Johnson, 1964; Cordero, 1990).

In recent years the theoretical exploration of mimcry has focussed on the distinction of imperfect versus perfect mimics (Johnstone, 2002; Sherratt 2002). The basic idea is that imperfect mimicry can be sufficient and might even provide better protection against predators because the imperfect mimics resembles more different models. In many zygopteran genera such as *Enallagma, Ceriagrion* and *Coenagrion* the so called andromorphs resemble the colouration of the males only, hence they are potentially imperfect mimics, in genera such as *Nehalennia* and *Ischnura* males and females do not differ in black patterning (e.g. Askew, 2004) and the

andromorphs could be perfect mimics. Johnson (1964; 1966) showed for *I. damula* and *I. demorsa* and Cordero (1990) and Andres and Cordero (1999) for *I. graellsii* and *C. tenellum* that these colour polymorphisms are genetically determined, and this is most likely the case for all coenagrionid species.

Several hypotheses and theories exist to explain the evolution and maintenance of this female colour polymorphism (Table 1). Most of these hypotheses as well as recent modelling approaches (Sherratt, 2001; Svensson *et al.,* 2005) are based on the idea that females are mimicking males. The 'learned mate recognition' hypothesis (Fincke, 1994 and 2004) still uses the terms 'andro'- and 'gynomorph/-chrome, however it is based on the idea that males prefer to mate with the most common morph. Furthermore, most studies either investigated the differences in the male's response to the female morphs (models or tethered animals) or the differences in male responses towards males, based on the assumption that 'androchrome' females mimic males. For example Gorb (1998) investigated and compared the male response towards 'androchrome' and 'gynochrome' female models, but did not compare the male response to either female morph with the response to a male model.

We feel that some of the explanations for female colour polymorphism may be confounded because they do not consider the differences between human and insect vision (Fig. 1). This is important when realizing that the 'male mimic' theory is based on the idea of an 'andromorph' female, as distinguished by human eye. Moreover, in many species this resemblance is restricted to the colour, in coenagrionids usually blue, but there is not necessarily a resemblance in the prominent black patterns. Another potential shortcoming with the explanations revolving around male mimicking is that they do not provide an explanation for species with several 'gynochrome' female morphs such as *Coenagrion pulchellum.*

Table 1: Hypotheses explaining the maintenance and/or evolution of colour polymorphisms female damselflies.

year	author	species	females similar to males in black patterning?	results	Based on male mimic?
1874	Darwin	*Ischnura spp.*	yes	Females look like males, probably driven by sexual selection	yes
1906	Ris	*Nehalennia speciosa*	yes	Different colours provide sun protection	no
1964, 1966, 1975	Johnson	*Ischnura damula,* *Ischnura demorsa*	yes yes	Reproductive isolation vs higher predation risk, shows genetic basis	yes
1985	Robertson	*Ischnura ramburi*	yes	Avoidance of time-consuming supernumerary matings balances the costs of relative greater predation for andromorphs	yes
1987	Hinnekint	*Ischnura elegans*	yes	At high male densities andromorph females avoid unnecessary matings while at low densities they have a greater risk of not mating at all.	yes
1988	Conrad and Pritchard	*Argia vivida*	yes	Thermoregulation, males do not prefer one morph, females do not differ in mating behaviour	no
1992	Cordero	*Ischnura graellsii*	yes	Avoiding male harassment, supposes differences in larval nutrition	yes
1994	Fincke	*Enallagma hageni* *Enallagma boreale*	no no	Null hypothesis: polymorphism neutral with respect to natural or sexual selection	
1999	Miller and Fincke	*Enallagma ebrium* *Enallagma civile*	no no	Learned mate recognition males prefer the most common female phenotype	no
1999	Van Gossum *et al.*	*Ischnura elegans*	yes	Habituation hypothesis	no
2001	Sirot and Brockmann	*Ischnura ramburi*	yes	Sexual interaction affects the morphs differently, no net costs for gynomorphs	yes
2001	Sherratt	Model		New male mimic	yes
2001	Sherratt and Forbes	Model		Evolution of male colour as sex-related warning, females mimicking this aposematism	yes
2001	Van Gossum *et al.*	*Ischnura elegans*	yes	Andromorphs mimic male behaviour	yes
2002	Andres *et al.*	*Ceriagrion tenellum*	no	Frequency dependent selection on colour polymorphism	yes
2003	Sirot *et al.*	*Ischnura ramburi*	yes	Signal detection hypothesis	yes
2004	Fincke	Model		Learned mate recognition, speciation via sexual signalling	
2004	McKee *et al.*	*Coenagrion puella* *Xanthocnemis zealandica*	no	Male response to andro- and gynomorph females does not differ, recognition could also be size and behaviour dependent.	yes in colour
2004	Miller and Fincke	*Enallagma aspersum* *Enallagma civile*	no no	Learned mate recognition	

Most current hypotheses are based on the assumption that ‘androchrome’ females have male attributes, at least to male damselflies. In this study we

investigate whether the patterning and colours of ‘androchrome’ females differ from males by analysing them using image analysis and RGB colour analysis. The study species is *Coenagrion puella*, where the females only resemble the male colour, not the black patterning and hence are a representative of the species group that show imperfect male mimicry. This approach circumvents the bias caused by human vision. In short, we ask whether ‘androchrome’ females are only ‘androchrome’ to the human eye, and not to male damselflies.

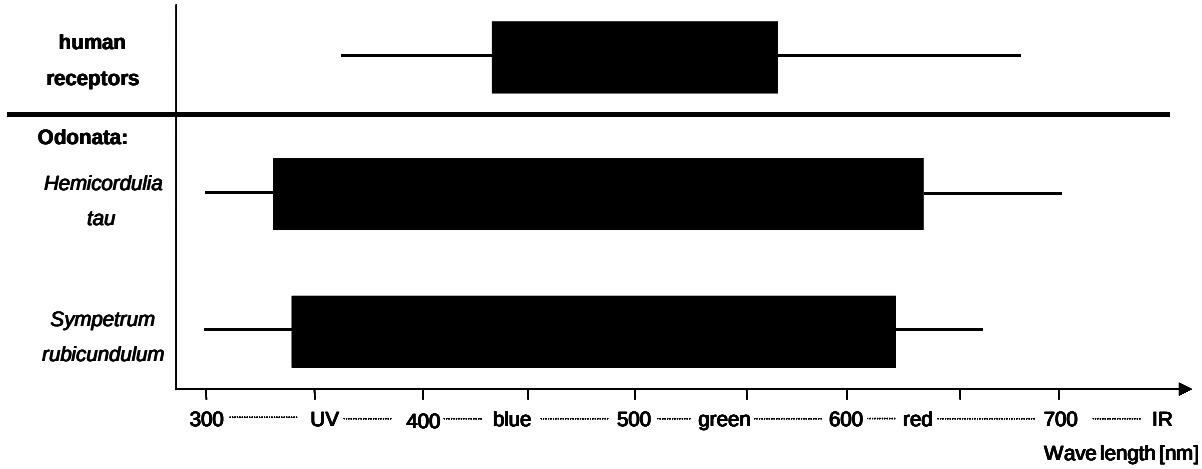

Figure 1: Here the number of receptors (height of bar), their range (width of line) and peaks (width of bar) are given for humans and Odonata, showing that Odonata have a wider range and two more receptors than we have. This allows them a better vision in the UV but also in red where some of the differences were found. Furthermore the receptors between 400 and 550nm, coding for the blue-green are closer together in Odonata than in humans, which again might explain why we find it hard to distinguish between blue females and males. Details for receptors compare Briscoe and Chittka (2001) and references within.

Methods

In *Coenagrion puella* (Coenagrionidae, Zygoptera) three female morphs have been described (Sternberg, 1999). Males of this species are light blue; blue females are consequently termed ‘androchrome’, and the ‘gynochromes’ are green. An

intermediate blue-green female morph also occurs (Sternberg, 1999; and pers. observation) (Fig. 2). As this intermediate morph also was found, when we collected teneral individuals and kept them to maturity, we assume this is a truly third morph and not depending on aging.

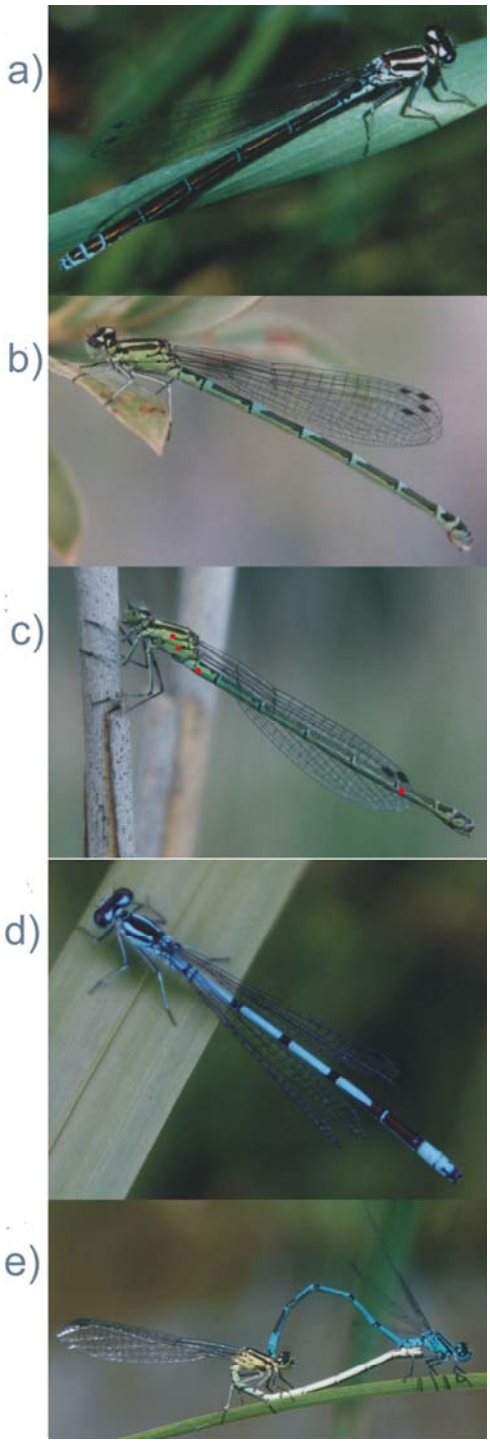

Figure 2: 1) – 3) show the female morphs of *C. puella*, 1) blue, 2) blue-green, 3) green, here red spots indicate where RGB measurements were taken, additionally head spots were measured, 4) shows a male and 5) shows a copulation wheel with a green female.

In summer 2003 (mid May to mid July) images were taken of the damselfly *C. puella* from two different populations near Braunschweig, Lower Saxony, Germany. Population 1 is a well studied population (Rolff and Martens, 1997; Braune and Rolff, 2001) more than fifteen years old, while population 2 was from a three year old pond (G. Joop, unpublished data). The populations were about 4 km apart and separated by woodlands and a motorway. Watts et al. (2005) could show for *C. mercuriale* that this are barriers hardly to cross, therefore we expect them to have no exchange with each other. To exclude aging effects, which might lead to colour changes, we only collected individuals with intact wings. Furthermore individuals with no watermites but their scars were excluded as this indicates that this animal has mated at least once (Rolff and Martens, 1997).

Images were taken of all three female morphs as well as males (20 blue females, 64 green and intermediate females and 110 males) (Minolta 500 sc, Fuji film sensia 200) on the same day as individuals were collected. Pictures were taken under standard light conditions (Philips new generation 58W/830, with 28lux on the spot the damselfly was fixed on) after chilling the individuals on ice. This was necessary, as blue odonate males may be capable of thermoregulatory colour change (May, 1979) and therefore a constant low temperature should reduce this variance. To test whether this cooling had any effect on the differences in colouration we also took images of individuals at ambient temperatures. To assess possible variation in light quality all images were taken and analysed with reference to a panel of colour standards. All slides were developed in the same lab and digitised using the Nikon coolscan III slide scanner. Colours were analysed using the R(ed)G(reen)B(lue) tool of Adobe Photoshop 6.0 (see Fitze *et al.,* 2003, our data not transformed to HSB, as we are interested in insect vision and HSB is adapted for human eyes (Fleishman and Edler, 2000)). Measurements were taken on five different spots of the damselfly body (head spots, 2 x thorax, second and seventh abdominal segment, see Fig. 2) and the mean of R, G and B calculated. The area of black was analysed using image analysing software (Optimas 6.1, Optimas corporation). Additionally we took UV images of the morphs in 2004 (4 blue, green and intermediate females, 4 males) (UV-pass filter by B+W, Ilford Delta 3200 professional black and white film) from population 1 to investigate potential differences in UV reflectance or absorbance.

Prior to analysis we ranked the individuals in one of four colour classes (male; blue, green or intermediate female) according to our vision. These colour classes and the population of origin (1 or 2) were used as fixed factors in the following 2-way MANOVA (Pillai's Trace), with total R, G and B and % black area (arcsin

transformed, Sokal and Rohlf, 1995) as dependent variables. All analyses were performed in SPSS 12.01.

Results

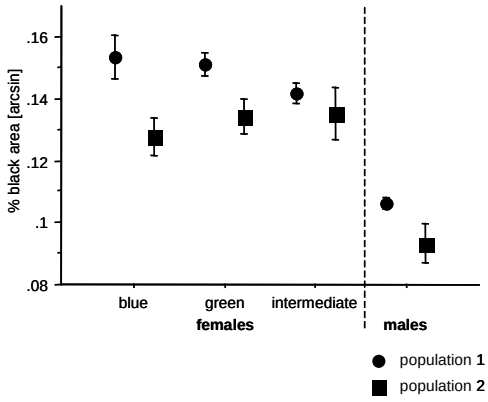

Figure 3: Percentage (%) black area in the female morphs and males of *C. puella* for two different populations. SE is given.

The colour classes (MANOVA: F=18.305, df=12, p<0.001) and populations (MANOVA: F=4.532, df=4, p<0.001) differ significantly in black pattering and colour composition (RGB). The interaction between colour class *x* population is not significant (MANOVA: F=1.040, df=12, p=0.410) and as shown in Figs 3 and 4, both populations show similar patterns. This indicates that acting selection might not be population dependent and underlying general differences might be caused by variation in e.g. condition.

Table 2: ANOVA table for differences in RGB and black pattern.

Source	Dependent variable	SS	df	F	p
Colour class	red content	6151.987	3	25.480	0.000
	green content	497.670	3	0.841	0.473
	blue content	40523.819	3	73.159	0.000
	% black	0.040	3	29.055	0.000
population	red content	130.115	1	1.617	0.205
	green content	188.026	1	0.954	0.330
	blue content	74.593	1	0.404	0.526
	% black	0.005	1	10.689	0.001
Colour class x population	red content	13.785	3	0.057	0.982
	green content	411.769	3	0.696	0.555
	blue content	1518.790	3	2.742	0.044
	% black	0.001	3	0.517	0.671

In terms of percentage black, the blue female morph of *C. puella* groups together with the other female morphs and all female morphs differ significantly from males (Table 2 and pair wise comparisons each female morph: males $p<0.0001$). Therefore, based on black patterns, the blue females differ considerably from males but not from other females.

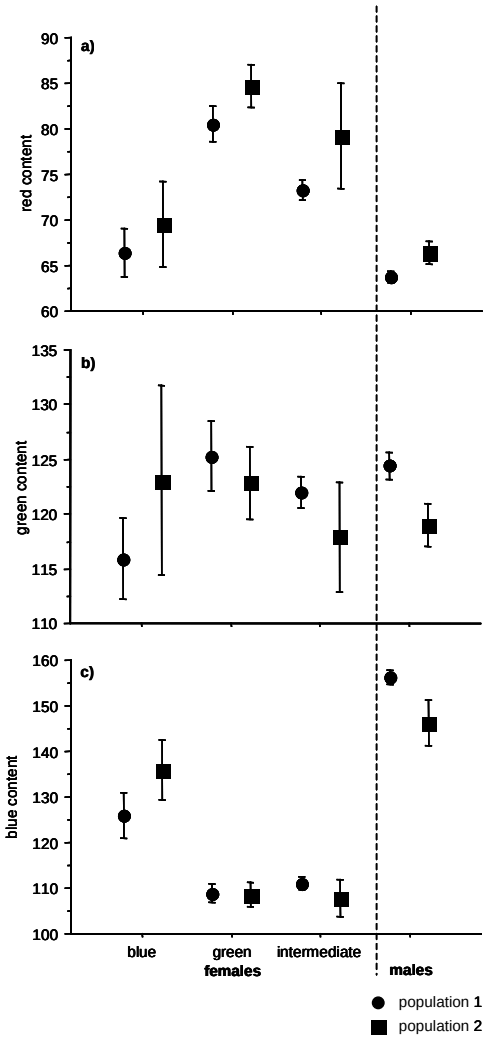

Figure 4: a) Red, b) green and c) blue content in the female morphs and males of *C. puella* for two different populations. SE is given.

The colour class result confirms our definition of colour classes. Blue females differ in their red content from the other females but not from males (Table 2 and pair wise comparison blue females: males $p=0.254$). They differ from both in their blue content (Table 2 and pair wise comparison all $p<0.0001$), while no differences were found for green content (Table 2). The colour analyses for the individuals measured in the field with mean day temperature as the covariate showed the same results (MANOVA: $F=18$, $df=6$, $p<0.001$). This suggests that blue females show a different blue when compared to males. In this analysis we also found a significant influence of mean day temperature (MANOVA: $F=1046$, $df=3$, $p<0.001$) and a significant interaction between mean day temperature and colour class (MANOVA: $F=3.18$, $df=3$, $p=0.005$), indicating that the colours change with temperature and that these changes go in different directions depending on colour class. Therefore it is unlikely that an optimal temperature at which colours should be measured can be found. We conclude that black patterning

might be a stronger female attribute than the colour differences as to our knowledge all animals are capable of detecting black contrasts.

Discussion

Our results are only partly consistent with the widely held view of ‘male-mimicking’ as an explanation for the blue colouration in female *C. puella,* a species with imperfect male mimics. ‘Male-mimicking’ seems to be an effect of human vision, as we cannot perceive the colour differences between blue females and males. Moreover, the hypothesis of male mimicking ignores the prominent black patterns: here all female morphs group together and differ significantly from males. However, one result is still consistent with the idea of imperfect male mimicking: the red contents of males and blue females where not distinguishable with our colour analysis. Insects have a specific red receptor (Fig. 1), so future research should explore this possibility.

UV vision in insects is mainly used as an explanation for their better, or rather different, visual impression of their environment. However, none of the UV images we have taken detected UV reflectance, which confirms the results of Hilton (1986). Therefore mate recognition is unlikely to be based on UV signals in *C. puella*.

That males can distinguish between the female morphs in *Coenagion puella* is suggested by the study of Gorb (1998). He used dead males and dead females of two colour morphs as models, thereby excluding behavioural differences, for choice experiments. These models were presented to free living males at a pond and the male responses were monitored. The responses differed between original male and female models and also between the two female morphs, but it was not tested

whether the response to blue females differed from that towards males. We reanalysed the data for males and females (Gorb, 1998; table II, unfortunately the presented data are not independent and therefore caution has to be applied to interpret the data), using a contingency table. We found over-all differences in male response to males, blue females and green females ($p<0.0001$). In pair-wise comparisons, we found that male responses towards blue females and males differed ($p<0.0001$) as it did between blue females and green females ($p<0.0001$) and green females and males ($p<0.0001$). Therefore, male *C. puella* are capable of distinguishing between males and blue females, even though in the tested population the response was strongest towards green females. Furthermore, Gorb (1998) suggested that black patterns were most important for mate recognition, which supports our idea of blue females in *C. puella* being recognizably female to males and is consistent with our data on black patterning (see Miller and Fincke, 1999).

These results do not address issues about behavioural differences between the female morphs, one morph might reject mating attempts more vigorously or fly differently and therefore might be harder to be recognized as female by males. Some observations (e.g. Gorb, 1998; Andres *et al.,* 2002; McKee *et al.,* 2004), especially on tethered females, found that males prefer the least male-like morph, but this could be due to behavioural changes depending on the experimental design. McKee *et al.* (2004) reported different results for tethered and free ranging females of *C. puella*: if tethered, males preferred the ‘gynochrome’ females but for released females they found no male preference for one of the female morphs. This indicates there might be no behavioural differences between the female morphs or that the behaviour of the blue females is so typically female that it helps the males recognise them as female. This is contrasting observations in ischnuran damselflies (Robertson, 1985; Cordero, 1989; Van Gossum *et al.,* 2001; Sirot *et al.,* 2003), where females

behaviourally mimicking males have been observed. As in ischnurans females are also more similar to the males in their black patterning we may have to distinguish between this two groups of female colour polymorphism within the coenagrionids (Table 1): genera in which females obviously differ from males in black patterning and genera where the sexes are similar in black content and patterning. The colour patterns in species where females mimic males perfectly such as *Ischnura*, however, to the best of our knowledge have not been investigated yet. A recent study over several seasons has revealed changes in morph frequencies of *Ischnura elegans*, a perfect mimic, that are consistent with the idea of sexual selection as a driver for the polymorphism (Svensson *et al.,* 2005).

Even though the problems of anthropomorphism are widely recognised in the study of animal behaviour (see Kennedy, 1992 as a general review), they seem to be difficult to overcome. Computer analysis provides a neutral tool for at least some of the problems and are widely used e.g. in the analysis of bird colouration. However, most computer programs are originally designed for graphics and therefore for human vision (Fleishman and Endler, 2000). Additionally we have to be more careful about transferring conclusions from one genera or even species to another. Male mimic in coenagrionids was proposed for ischnurans only in the first place and only later used to explain colour polymorphism in other genera such as *Enallagma* or *Coenagrion*.

We feel that our approach can be used to resolve the controversies of the evolution of female colour polymorphism, as it offers a tool to estimate the mimicry patterns and thereby allows to refine modelling assumptions. This method is also applicable to species with perfect male mimics such as *Ischnura*. If sexual selection is not the driving force of the colour polymorphism (see also Sirot and Brockmann, 2001) than possible explanations for the evolution and maintenance of such colour

polymorphisms could be differences between the morphs that are not related to sexual selection such as survival or larval life history (Andres and Cordero Rivera, 2001; Cordero, 1992). Finally, males of many coenagrionid species have very similar colouration at least to the human eye and often species co-occur (Rolff, 2002 as an example). It would be intriguing to explore these similarities using the experimental approach presented above in the context of male aposematism, a warning colouration to avoid costly harassment as proposed by Sherratt and Forbes (2001) for an intra-specific scenario.

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References

Andolfatto, P., Scriber, J. M. and Charlesworth, B. (2003) No association between mitochondrial DNA haplotypes and a female-limited mimicry phenotype in *Papilio glaucus*. *Evolution,* **57**, 305-316.

Andres, J. A. and Cordero, A. (1999) The inheritance of female colour morphs in the damselfly *Ceriagrion tenellum* (Odonata, Coenagrionidae). *Heredity,* **82**, 328-335.

Andres, J. A. and Cordero Rivera, A. (2001) Survival rates in a natural population of the damselfly *Ceriagrion tenellum*: effects of sex and female phenotype. *Ecol. Entomol.,* **26**, 341-346.

Andres, J. A., Sanchez-Guillen, R. S. and Cordero Rivera, A. (2002) Evolution of female colour polymorphism in damselflies: testing the hypothesis. *Anim. Behav.,* **63**, 677-685.

Askew, R. R. (2004) *The dragonflies of Europe*. Harley Books, Colchester.

Braune, P. and Rolff, J. (2001) Parasitism and survival in a damselfly: does host sex matter? *Proc. R. Soc. Lond. B,* **268**, 1133-1137

Briscoe, A. D. and Chittka, L. (2001) The Evolution of color vision in insects. *Ann. Rev. Entomol.,* **46**, 471-510.

Conrad, K. F. and Pritchard, G. (1988) Female dimorphism and physiological colour change in the damselfly *Agria vivida* Hagen (Odonata: Coenagrionidae). *Can. J. Zool.,* **67**, 298-304.

Cordero, A. (1990) The inheritance of female polymorphism in the damselfly *Ischnura graellsii* (Rambur) (Odonata: Coenagrionidae). *Heredity*, **64**, 341-346.

Cordero, A. (1992) Density-dependent mating success and colour polymorphism in females of the damselfly *Ischnura graellsii* (Odonata: Coenagrionidae). *J. Anim. Ecol.,* **61**, 769-780.

Darwin, C. (1873) *The Descent of man.* Murray, London.

Fincke, O. M. (1994) Female colour polymorphism in damselflies: failure to reject the null hypothesis. *Anim. Behav.,* **47**, 1249-1266.

Fincke, O. M. (2004) Polymorphic signals of harassed odonates and the males that learn them support a novel frequency-dependent model. *Anim. Behav.,* **67**, 833-845.

Fitze, P. S., Kölliker, M. and Richner, H. (2003) Effects of common origin and common envirnoment on nestling plumage coloration in the Great Tit (*Parus major*). *Evolution,* **57**, 144-150.

Fleishman, L. J. and Endler, J. A. (2000) Some comments on visual perception and the use of video playback in animal behaviour studies. *Acta Ethol.,* **3**, 15-27.

Gamble, S., Lindholm, A. K., Endler, J. A. and Brooks, R. (2003) Environmental variation and the maintenance of polymorphism: the effect of ambient light spectrum on mating behaviour and sexual selection in guppies. *Ecol. Letters,* **6**, 463-472.

Gorb, S. N. (1998) Visual cues in mate recognition by males of the damselfly, *Coenagrion puella* (L.) (Odonata: Coenagrionidae). *J. Ins. Behav.,* **11**, 73-92.

Hilton, D. F. J. (1986) A survey of some Odonata for ultraviolet patterns. *Odonatologica,* **15**, 335-345.

Hinnekint, B., O, N. (1987) Population dynamics of *Ischnura E. elegans* (Vander Linden) (Insecta: Odonata) with special reference to morphological colour change, female polymorphism, multiannual circles and their influence on behaviour. *Hydrobiologia,* **146**, 3-31.

Johnson, C. (1964) The inheritance of female dimorphism in the damselfly, *Ischnura damula*. *Genetics,* **49**, 513-519.

Johnson, C. (1966) Genetics of female dimorphism in *Ischnura demorsa*. *Heredity,* **21**, 453-459.

Johnson, C. (1975) Polymorphism and natural selection in ischnuran damselflies. *Evol. Theo.*, **1**, 81-90

Kennedy, J. S. (1992) *The new anthropomorphism.* University Press, Cambridge.

May, M. L. (1979) Insect Thermoregulation. *Ann. Rev. Entomol.,* **24**, 313-349.

McKee, D., Harvey, L. and Sherratt, T. N. (2004) Behaviour of male Coenagrionid damselflies towards conspecific females at the water's edge (Zygopteran: Coenagrionidae). *Odonatologica,* **33**, 271-278.

Miller, M. N. and Fincke, O. M. (1999) Cues for mate recognition and the effect of prior experiences on mate recognition in *Enallagma* damselflies. *J. Ins. Behav.,* **12**, 801-814.

Miller, M. N. and Fincke, O. M. (2004) Mistakes in sexual recognition among sympatric Zygoptera vary with time of day and color morphism (Odonata: Coenagrionidae). *Internat. J. Odonatol.,* **7**, 471-491.

Ris, F. (1906) Farbenvarietäten der Agrionide *Nehalennia speciosa* Charp. *Mitteilungen der schweizerischen entomologischen Gesellschaft,* **11**, 159-165.

Robertson, H. M. (1985) Female dimorphism and mating behaviour in a damselfly, *Ischnura ramburi*: females mimicking males. *Anim. Behav.,* **33**, 805-809.

Rolff, J. (2002) Bateman's principle and immunity. *Proc. R. Soc. Lond. B*., **269**, 867-872.

Rolff, J. and Martens, A. (1997) Completing the life cycle: detachment of an aquatic parasite (*Arrenurus cuspidator*, Hydrachnellae) from an aerial host (*Coenagrion puella*, Odonata). *Canad. J. Zool.,* **75**, 655-659.

Sherratt, T. N. (2001) The evolution of female-limited polymorphisms in damselflies: a signal detection model. *Ecol. Letters,* **4**, 22-29.

Sherratt, T. N. (2002) The evolution of imperfect mimicry. *Behav. Ecol.*, **13**, 821-826.

Sherratt, T., N and Forbes, M., R. (2001) Sexual differences in coloration of Conagrionid damselflies (Odonata): a case of intraspecific aposematism? *Anim. Behav.,* **62**, 653-660.

Sirot, L. K. and Brockmann, H. J. (2001) Costs of sexual interactions to females in Rammbur's forktail damselfly, *Ischnura ramburi* (Zygoteran: Coenagrionidae). *Anim. Behav.,* **61**, 415-424.

Sirot, L. K., Brockmann, H. J., Marinis, C. and Muschett, G. (2003) Maintenance of female-limited polymorphism in *Ischnura ramburi* (Zygoptera: Coenagrionidae). *Anim. Behav.,* **66**, 763-775.

Sokal, R. and Rohlf, F. (1995) *Biometry.* W.H. Freeman and Company, New York.

Sternberg, K. (1999) *Coenagrion puella*. In: *Die Libellen Badenwürttembergs, Kleinlibellen (Zygoptera)* (eds. Sternberg, K. and Buchwald, L.), 278-287. Verlag Eugen Ulmer, Stuttgart.

Sundberg, J. (1995) Parasites, plumage coloration and reproductive success in the yellowhammer, *Emberiza citrinella*. *Oikos,* **74**, 331-339.

Svensson, E. and Sinervo, B. (2000). Experimental excursions on adaptive landscapes: density-dependent selection on egg size. *Evolution,* **54**, 1396-1403.

Svensson E. I., Abbott, J. and Härdling, R: (2005) Polymorphism and Evolutionary Dynamics. *Am. Nat*., **165**, 567-576.

Vane-Wright, R. I. (1979) Towards a theory of the evolution of butterfly colour patterns under directional and disruptive selection. *Biol. J. Linn. Soc.,* **11**, 141-142.

van Gossum, H., Stoks, R., Matthhysen, E., Valck, F. and De Bruyn, L. (1999) Male choice for female colour morphs in *Ischnura elegans* (Odonata, Coenagrionidae): testing the hypotheses. *Anim. Behav.,* **57**, 1229-1232.

van Gossum, H., Stoks, R. and De Bryn, L. (2001) Frequency-dependent male mate harassment and intra-specific variation in its avoidance by females of the damselfly *Ischnura elegans. Behav. Ecol. Sociobiol.*, **51**, 69-75.

Watts, P. C., Rouquette, J. R., Saccheri, J., Kemp, S. J. and Thompson, D. J. 2004. Molecular and ecological evidence for small-scale isolation by distance in an endangered damselfly, *Coenagrion mercuriale*. *Molecular Ecology*, **13**, 2931-2945.

Wente, W. H. and Phillips, J. B. (2003) Fixed green and brown color morphs and a novel color-changing morph of the pacific tree frog *Hyla regilla*. *Am. Nat.,* **162**, 461-473.

Chapter II

‚[Gender] are simply life history adaptations that maximize individual fitness through male and/or female function in light of various constraints.'

(Rhen and Crews 2001)

Plasticity of immune function and condition under the risk of predation and parasitism

Abstract

Ecological immunology attempts to elucidate the causes of the large variation in immunity and resistance observed in natural populations. Here we report on a novel experiment that investigated how the risks of parasitism and predation altered investment in immunity and condition in insects during larval development. The study organism is the damselfly *Coenagrion puella,* the parasite is a water mite and predators are encaged *Aeshna cyanea* dragonflies. Our experiments show that females increase their investment in a cellular as well as humoral component of the immune system in the presence of natural enemies. By contrast males do not show such alteration. However, males show altered condition under the risks of parasitism and predation. Our results highlight the importance of species interactions for the plasticity of immune function.

Introduction

In the wild organisms face multiple natural enemies, which is often a simultaneous occurence (Crawley, 1992; Sih *et al.*, 1998). Facing multiple enemies can result in trade-offs, such that avoiding one enemy results in a higher encounter rate with another enemy (Decastaecker *et al.,* 2002). However, only a few studies have been conducted studying the impact of multiple enemies simultaneously. These have focussed on behavioral as well as life history changes (e.g. Baker and Smith, 1997; Decastaeker *et al.,* 2002; Lass and Bittner, 2002; Eklöv and Werner, 2000). Our aim was to investigate the effects of multiple natural enemies on plasticity of immune function and condition (see Rolff and Joop, 2002; for the use of condition) in larval insects.

We concentrate on these traits because they are closely linked to fitness and to one another (Plaistow and Siva-Jothy, 1996; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003). Furthermore we included gender in our analysis. Gender differences are often neglected in such ecological studies. They have, however, been shown to be important (Rolff, 2002, Zuk and Stoehr, 2002, and references therein), as males and females take different routes to fitness (Bateman, 1948). To the best of our knowledge, only one experiment has studied the impact of multiple enemies with regard to immunity (Rigby and Jokela, 2000). Our study amplifies the study by Rigby and Jokela (2000) in four important aspects:

(i) animals are exposed to a perceived risk of parasitism and predation respectively

(ii) allocation during larval development is looked at

(iii) we include condition

(iv) we examine gender differences as opposed to hermaphrodites

Insects with aquatic larvae and terrestrial adults undergo an extreme habitat shift (Johansson and Rowe, 1999; Plaistow and Siva-Jothy, 1999). During this habitat shift damselflies are probably at the most vulnerable stage of their life history (Corbet, 1999). Differences in immunity and condition caused earlier in their larval stage should become more pronounced and determine the success of the habitat shift. We used the damselfly *Coenagrion puella* as the study organism, the predators were larvae of the dragonfly *Aeshna cyanea* and the parasites were larvae of the water mite *Arrenurus cuspidator*. Insect predators have been demonstrated to influence the choice of microhabitats (Suhling and Lepkojus, 2001), reduced survival rates, and changes in activity in larval damselflies and dragonflies (Stoks *et al.,* 1999). The presence of predators imposes stress that can cause malnutrition (Johansson and Leonardsson, 1998) or reduce digestive efficiency (Stoks and McPeek, 2003). The risk of predation can easily be exerted with caged predators (Koperski, 1997). Parasites also affect life history and behaviour of their host (Moore, 2002). Water mites have been shown to reduce, amongst other traits, the survivorship of adult damselflies (Braune and Rolff, 2001). Water mite larvae settle on damselfly larvae in the final instar but do not parasitize until the larvae emerge (Rolff, 2001). This enabled us to manipulate the risk of parasitism of adult dragonflies. Larval water mites induce behavioural changes in larval damselflies (Baker and Smith, 1997; Leung *et al.,* 1999). Such changes have been interpreted as parasite avoidance behaviour (Baker and Smith, 1997). Hence larval dragonflies are capable of perceiving the risk of parasitism of their adult stage. We concentrated on the late larval instars of the damselflies, as this is the time when they share their microhabitats with the predator (Inden-Lohmar, 1997) and parasite (Mitchell, 1967) used in this study.

Innate immunity has been defined “a set of disease-resistance mechanisms that are not specific to a particular pathogen” (e.g. due to anatomy, physiological, phagocytotic, and inflammatory mechanisms, (Goldsby *et al.,* 2000)). We studied two different components of the insect innate immune system: cellular defence and an important part of humoral defence, the Phenoloxidase cascade (Gillespie *et al.,* 1997). Hemocytes, insect blood cells, play an important role in insect immune defence. They recognise pathogens, are involved into encapsulation and phagocytosis (Kurtz *et al.,* 2000). There is also good evidence that hemocyte count is positively related to successful immune defence (Fellowes and Godfray, 2000; Kurtz *et al.,* 2000). Phenoloxidase (PO) is an enzyme present in most arthropods' hemolymph and cuticle (Sugumaran, 2002). PO catalyses the production of melanin, which takes part in wound repair and encapsulation. Using *Drosophila* mutants, Braun *et al.*, (1999) demonstrated that PO activity is positively correlated with resistance against a variety of pathogens. We measured condition by assessing different physiological traits (Rolff and Joop, 2002): fat content, muscle mass and skeletal size, all of which are related to fitness in damselflies (Marden, 1989; Plaistow and Siva-Jothy, 1996, Sokolovska *et al.*, 2000).

Here, we examine the notion whether insects alter their allocation to immune function and condition in the presence of natural enemies. Because of the parasites life cycle and the caging of the predators we are able to manipulate the perceived risk of parasitism and predation.

Material and Methods

Study organisms

The target species *C. puella* (Odonata, Insecta) is a common and well studied (e.g. Corbet, 1999; Rolff, 2001) damselfly of northern and central Europe, usually occurring in small ponds. Larvae hibernate in later instars. Shortly before emergence the damselfly larvae move to shallow water regions. The damselfly larvae were caught in the first two weeks in April 2001 in the area "Klei" near Braunschweig (Lower saxony, Germany, 52°21'N, 10°35'E), when they were in the last three instars.

A. cuspidator (Hydrachnellae, Acarina) is a common ectoparasite on damselflies in central Europe (Rolff, 2001). After the damselfly's emergence the mites climb from the exuvia to the newly emerged adult. The mite larvae then pierce the cuticle and feed on hemolymph and tissues. During oviposition mites from male and female damselflies detach and complete their life cycle (Rolff, 2001).

A. cyanea (Aeshnidae, Odonata) is a widespread and abundant dragonfly species in Europe, it's habitat overlaps strongly with that of *C. puella* (Inden-Lohmar, 1997). The larval stage lasts two years (Inden-Lohmar, 1997), last instars of *A. cyanea* can predominantly be found close to the water surface, an area which is preferred by late instars of *C. puella* as well.

Experimental design

All damselfly larvae were held under the same temperature, feeding conditions and light. We used a full factorial design with the following non-lethal risk treatments, each replicated four times with 25 *C. puella* larvae per tank: (a) control (b) with parasite (approximately 400 *A. cuspidator* larvae) (c) with predator (one caged *A.*

cyanea larva) (d) with parasite and predator (later on referred to as two risks). Unfortunately one predation replicate got contaminated with *A. cuspidator*. This tank was therefore excluded from the analyses.

Each of the sixteen tanks was filled with gravel to a height of 2.5 cm and with tap water to the height of 16 cm (total water volume of 6.7 litres). Each tank was initially stocked with 25 damselfly larvae, resulting in a density of about 250 larvae per m^2 which is comparable to natural conditions (Banks and Thompson, 1987). As the larvae were in different instars their head width was measured, and instars distributed equally among tanks, but at random within instars. The tanks were placed randomly. The experiment started on the 24th of April 2001.

Aeshnid larvae in the ante-penultimate stadium F–2 were caged (17.6 x 7.5 x 7.5 cm) to avoid predation (Koperski, 1997). The cages were covered with mesh and gauze (mesh wide = 0.5 mm). This allowed water to flow through the cage and the damselfly larvae to detect the predator visually and chemically (Koperski, 1997). To make sure that the presence of a cage had no influence on the damselfly behaviour cages were placed in each tank. Further furniture was a plastic plant and a climbing structure.

Johansson (1996) showed that feeding six *Daphnia* spec. to a coenagrionid damselfly larva every other day guarantees feeding to satiation. If the damselflies are fed to satiation, they may not be resource limited. Therefore they were fed at a lower level. Because of the rising temperatures from April to May the damselflies were fed in changing intervals as can be seen in table 1. *A. cyanea* were fed biweekly with *Chironomus* spec. *A. cuspidator* larvae do not feed during the free-living stage.

Table 1: Feeding levels during the experiment

duration	average max. room temperature	frequency	number of *Daphnia* per damselfly
24.04.-30.04.2001	13.14°C	every third day	4
01.05.-12.05.2001	14.46°C	every second day	4
13.05.-05.07.2001	18.35°C	first day,	4
		second day, alternating	2

From the 17th May 2001 an emergence structure was presented to the damselfly larvae, during the first 19 days for two hours per day (10.00-12.00 or 12.00-14.00), and in the following period for four hours per day (10.00-14.00). When a larva climbed out of the water it was caught with tweezers and put separately into ice-cold water to prevent emergence (see Rolff, 1999). This slows down all physiological reactions. We did not collect the damselflies after emergence because after eclosion the mites become parasitic and interfere with the immune system of the host. The animals were stored until 14:00 h in the cold water on ice and the following measurements were conducted.

Morphometric and physiological measurements

The larvae were taken out of the water and gently dried with paper tissue before being placed in a micro centrifuge tube and kept on ice for chilling. Their fresh weight was measured to the nearest 100 µg (Mettler PM 480 DeltaRange). After chilling them on ice again blood samples were taken (see below), the damselflies were sexed and the head width measured. A *camera lucida* and a digital vernier calliper (Mitutoyo, Digimatic) were used for measuring head width. Head width is a common indicator for size in damselflies (Banks and Thompson, 1987).

Following the protocol of Barnes and Siva-Jothy (2000), haemolymph extracts were obtained by perfusing with 0.3 ml cacodylate buffer (0.01M Na-Coc, 0.005M $CaCl_2$). The last abdominal segment was cut off and the buffer injected into the ventral side of the first thoracic segment (syringe: Beckton Dickinson, Micro-fine, 0.3 ml, 0.33 x 12.7 mm). The sample was aliquoted for haemocyte counting and assaying phenoloxidase activity (see below). The bled larvae were frozen at –90°C and kept for analyses of fat content and muscle mass.

20 µl of each blood sample were pipetted into one well of a multi-well slide coated with Poly-D-Lysin (0.01 mg per 1 ml) (M.T. Siva-Jothy, pers. comm.). The slides were incubated in a humid chamber at room temperature for 1 hour. 1 µl DAPI (4,6-Diamidino-2-phenylindol Dihydrochlorid) solution was added as a fluorescent stain and the slides were left to dry in a dark box over night. Haemocytes were counted using a Leitz-Diaplan microscope with an image analyser (optimas 6.1, Optimas corporation). As it was not possible to count a whole well, three pictures were taken at random and the average cell counts determined (magnification x 20) (Ryder and Siva-Jothy, 2001).

To analyse PO activity the samples were thawed in ice water and the cell walls removed via centrifugation (4°C, 6500 rpm, 15 minutes (Eppendorf centrifuge 5417R)). 0.2 ml of the supernatant was added to 0.6 ml L-DOPA (10 mM in Na-Coc buffer). Readings of the absorbance were recorded every minute for a duration of 30 minutes at 30°C and a slope calculated from these, using SWIFT II analysis software (see Barnes and Siva-Jothy, 2000 for details).

The frozen larvae were thawed and freeze-dried for 24 h (LSL SECFROID, LYOLAB B), their dry mass was measured to the nearest 10 µg (Mettler AE 160). Fat extraction followed the protocol of Plaistow and Siva-Jothy (1996, and references within), using a Soxhlett extractor, for eight hours of continuous reflux (Plaistow and Siva-Jothy, 1996; Rolff and Joop, 2002). After fat extraction the damselflies were freeze-dried again, and re-weighed. The calculated difference between dry-weight and fatless-weight was taken as fat content.

The fatless and dried bodies were covered with 0.35 M Sodium hydroxide (NaOH) for 24 h at room temperature in 1.5 ml micro centrifuge tubes. After soaking, the hydroxide was removed, replaced with distilled water for an hour, and the bodies freeze-dried for 24 h (see Marden, 1989). The muscle mass was calculated from the

difference between the fatless mass and the muscle-less mass. Cuticle weight is the remaining weight after fat and muscle extraction have been done.

Statstics

We analysed the data using full-factorial ANCOVAs. The factors were predation and parasitism and tank means of the traits under study were used as response variable. Size and day of emergence were entered as co-variates. Day of emergence measures the time at which individuals within one tank, where on average exposed to treatments. Data were analysed separately for gender (see below for looking at gender differences), because physiological differences were expected a priori (e.g. Kurtz *et al.*, 2000, Braune and Rolff, 2001). The other physiological traits, wet weight, dry weight, cuticle weight, fat content, and muscle mass were highly correlated. Hence they were collapsed into two variables using a principal component analysis based on the correlation matrix (which accounts for different scaling of the data). We ran separate PCA for males and females, because of the *a priori* known gender differences. Subsequent tests were performed using the PC as response variables. Parasite x Predator interactions were removed if non-significant.

Sexes differ in immune traits in *C. puella* (G. Joop, unpublished data). However, our prime interest was not the gender differences *per se* but to test whether the plasticity of immune function is different in different environments, i.e. treatments. We first calculated differences in immune trait expression between male and female tank means. Then we ran a model with these calculated differences as response (dependent) variables and the risks of parasitism and predation as factors. This was not possible for the other physiological traits, because different traits loaded in a different manner on the principal components extracted.

Results

In the presence of parasites or predators alone females showed an increased hemocyte density but not in the two risk treatment (see Table 2, Fig. 1).

Table 2: ANCOVA results for haemocytes, PO-activity and condition separate for gender. Note that PC1 and PC2 for males are not the same as for females (see table 3)

	male			female		
source	**df**	**F**	**P**	**df**	**F**	**P**
haemocytes						
Predator	1	0.034	0.857	1	0.348	0.570
Parasite	1	1.190	0.301	1	1.586	0.240
Day of emergence	1	1.251	0.270	1	7.426	0.023
Head width	1	0.002	0.969	1	4.269	0.069
Predator : Parasite				1	9.922	0.033
Residuals	10			9		
PO						
Predator	1	0.691	0.427	1	3.478	0.095
Parasite	1	0.127	0.730	1	0.145	0.712
Day of ermergence	1	2.667	0.137	1	2.162	0.176
Head width	1	1.103	0.321	1	0.555	0.475
Predator : Parasite	1			1	7.319	0.024
Residuals	10			9		
PC1						
Predator	1	4.844	0.052	1	0.061	0.810
Parasite	1	5.837	0.036	1	0.721	0.416
Day of ermergence	1	3.640	0.085	1	1.583	0.237
Head width	1	0.529	0.484	1	1.562	0.240
Residuals	10			10		
PC2						
Predator	1	0.008	0.931	1	1.583	0.240
Parasite	1	5.879	0.038	1	0.379	0.553
Day of ermergence	1	0.514	0.491	1	0.688	0.428
Head width	1	3.754	0.085	1	0.537	0.482
Predator : Parasite	1	3.523	0.093	1	2.931	0.121
Residuals	9			9		

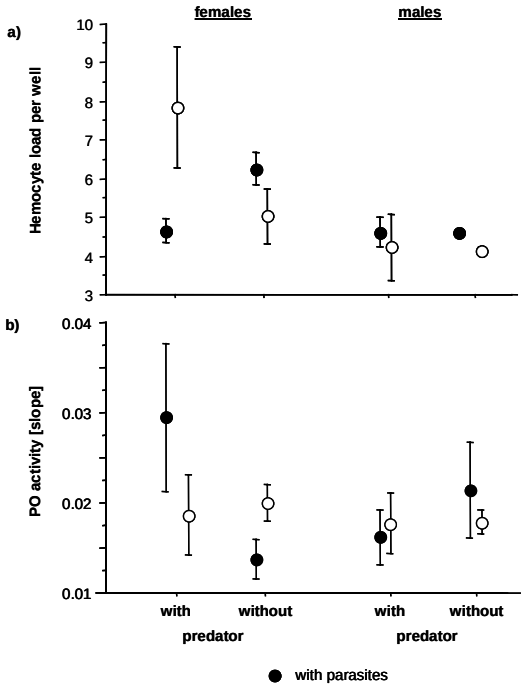

Figure 1: The effects of treatment on a) female and male hemocyte load (sqr-transformed) and b) on female and male PO activity (log-transformed). Error bars represent standard errors of the mean.

By contrast, male hemocyte density was not affected by the risks of parasitism and predation (Table 2, Fig. 1). The plasticity of male and female hemocyte density differed significantly across treatments (Predator x Parasite: $F_{1,9}$=13.4, p=0.005, response variable (female tank mean – male tank mean) see material and methods for the structure of the model).

Only in the two risks treatment females showed an increased PO activity (see Table 2, Fig. 1). The PO activity in males was not altered by treatment (Table 2, Fig. 1). Male and female plasticity in POactivity differed significantly (Predator x Parasite: $F_{1,9}$=6.9, p=0.028, see material and methods for the structure of the model).

Table 3: Factor Loadings of the physiological traits on the principal components. Note that separate PC were calculated for males and females.

	male		female	
trait	pca1	pca2	pca1	pca2
freshweight	0.166	0.847	0.847	-0.010
dryweight	0.859	0.489	0.853	0.492
cuticulaweight	0.876	0.028	0.088	0.951
fatcontent	0.073	0.916	0.651	0.475
musclemass	0.918	0.086	0.922	0.144

We extracted two principal components for males and females respectively (Table 3, Fig. 2). For males PC1 correlates highly positive with muscle mass, dry weight, and cuticle weight and explains 57.95 % of the variance. PC 2 correlates highly positive with fat content and fresh weight and explains 26.64% of the variance. Together PC1 and 2 explain 84.59% of the variance. Predators and parasites have an effect on PC1 such that condition is lower under the risk of predation (see Table 2, Fig. 2). PC2 (mainly related to fat content) is

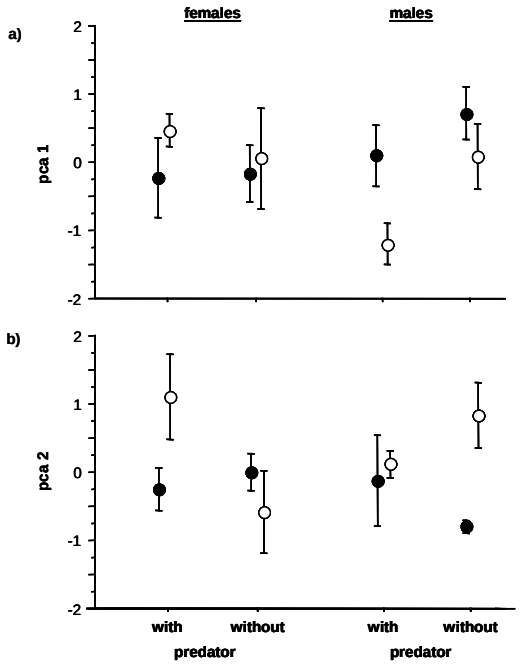

Figure 2: Treatment effects on condition for males and females as measured by a) PC1 and b) PC2. Note that both, PC1 and PC2 are estimated separately for females and males (see table 3). Error bars represent standard errors of the mean.

decreased in the presence of parasites.

For females PC1 correlates highly positively with muscle mass, dry mass, fat content and wet mass and explains 64.14 % of the variance. PC 2 correlates highly positive with cuticle mass and less strictly with fat content and dry mass and explains 18.20% of the variance. Together PC1 and 2 explain 82.34% of the variance. In females condition is neither altered by the risk of parasitism nor by the risk of predation (table 2).

Discussion

The results show that the perceived risks of predation and parasitism significantly alter components of immune function and condition of larval *Coenagrion puella*. The experimental set-up allowed for independent manipulation of parasitism and predation risks, as well as combining these two factors. Females altered the expression of the two immunological traits studied, hemocyte density and PO activity. Both were increased in the presence of natural enemies. However, hemocyte load was increased only in the presence of a single natural enemy. PO activity only increased in the simultaneous presence of both natural enemies. By contrast male immune function was neither influenced by the risk of predation nor the risk of parasitism. Furthermore females have a higher hemocyte density than males. This confirms findings in other insect species (see Kurtz *et al.,* 2000, Rolff, 2002, for references).

Even though both sexes are exposed to the same risks of parasitism and predation, respectively, their reaction differs. The increase in components of the immune system such as hemocyte numbers in the presence of predators can be

useful because these mechanisms are involved in wound repair (Sugumaran, 2002). Wounding occurs regularly in damselfly larvae (Baker and Dixon, 1986). Predators often remove lamellae from their prey without actually killing it (Stoks, 1999). Such wounds are potential sites for pathogen invasions. Why, however, do females invest either in PO or in hemocytes? And why do males not react to the presence of natural enemies in this way? One could postulate that females might have more plasticity in immune function, in the case of hemocytes simply because they have more than males. Females may also differ in their microhabitat use as well as in the efficiency of food digestion. Furthermore there are differences in life history trajectories, males maximize fitness by increasing the mating rate (Bateman, 1948) whereas for females the main predictor for fitness is longevity (Banks and Thompson, 1987). Male are more plastic in late larval instars (Baker *et al.,* 1992). Females put more weight on between emergence and sexual maturity than males (Anholt *et al.,* 1991). Because of the physiological analyses we were not in a position to investigate whether females trade-off immunity against for example fecundity later in life. We can, however, state that parasitized females produce smaller clutches (Rolff, 1999) and show higher mortality (Braune and Rolff, 2001) than parasitized males. Therefore there may be a greater selection pressure upon females to investment in immune components (Rolff, 2002). *C. puella does* not exhibit successful resistance against the water mite *A. cuspidator* (Rolff, 2001). However, because of the haemolymph loss and opportunistic infections, investment in immune function might pay off in the long term. This argument is based on the assumption that females need to invest more in immunity than males (Rolff, 2002; Zuk and Stoehr, 2002). All these reasons can explain why females invest more in immunity in the presence of natural enemies. However, why they increase the hemocyte number in the presence of one but not two

natural enemies, but increase PO activity in the simultaneous presence of predator and parasite (two risk treatment) is not known.

We expected condition to be lower in the presence of predators. It has been shown that in the presence of fish or invertebrate predators damselflies such as *Enallagma cyathigerum* (Koperski, 1997) or *Lestes sponsa* (Stoks *et al.,* 1999) decrease activity and digestive efficiency (McPeek *et al.,* 2001, Stoks and McPeek, 2003). In contrast to the immune traits, only males reacted to the presence of natural enemies. Males showed a decrease in condition, in the principal component (pc1) that mainly comprises muscle mass and dry weight, in the presence of predators. However, in the presence of parasites only, they show an ambiguous reaction. PC2, the component that is mainly related to fat content and fresh weight decreased. In contrast, PC1 increased. As survivorship after emergence depends on condition (Braune and Rolff, 2001), this shift in condition could be interpreted as adaptive. Males showed more plasticity in condition in the presence of predators or parasites than females. This might be explained by the fact that male coenagrionid dragonflies are more active in the last instars (Baker *et al.,* 1992). As activity is usually reduced in the presence of predator, the reduction in males condition could be stronger, even though there are currently no published data available on this issue.

The study of how plasticity of immune function is shaped in an ecological context, especially during the interaction with natural enemies, is only in its infancy (Rolff and Siva-Jothy, 2003). The few studies conducted so far have shown that social environment (Traniello *et al.,* 2002), habitat (Kurtz *et al.,* 2002), density (Wilson *et al*., 2001) and diet (Feder *et al.*, 1997) influence the expression of the immune response. However, it is still a long way from a thorough understanding of how the environment shapes the investment in immune function (Rolff and Siva-Jothy, 2003) and alters allocation rules (Worley *et al.,* 2003). Here we have experimentally

combined two major sources of variability in immune function, gender differences and interspecific interactions (Schmid-Hempel, 2003). We have shown that males and females differ in their investment in components of immunity and condition if they perceive risks posed by natural enemies.

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References

Anholt, B., Marden, J. and Jenkins, D. 1991. Patterns of mass gain and sexual dimorphism in adult dragonflies. *Can. J. Zool.,* **69**: 1156-1163.

Baker, R. and Dixon, S. 1986. Wounding as an index of aggressive interactions in larval Zygoptera (Odoanta). *Can. J. Zool.,* **64**: 893-897.

Baker, R., Forbes, M. and Proctor, H. 1992. Sexual differences in development and behaviour of larval *Ischnura verticalis*. *Can. J. Zool.,* **70**: 1161-1165.

Baker, R. and Smith, B.P. 1997. Conflict between antipredator and antiparasite behaviour in larval damselflies. *Oecologia* **109**: 622-628.

Banks, M. and Thompson, D.J. 1987. Lifetime reproductive success of females of the damselfly *Coenagrion puella*. *J. Anim. Ecol.* **56**: 815-832.

Barnes, A. and Siva-Jothy, M. 2000. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proc. R. Soc. Lond. B.,* **267**: 177-182.

Bateman, A. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**: 349-368.

Braun, A., Hoffmann, J.A. and Meister, M. 1998. Analysis of the Drosophila host defense in domino mutant larvae, which are devoid of hemocytes *Proc. Nat. Acad. Sci. USA***, 95**: 14337-14342.

Braune, P. and Rolff, J. 2001. Parasitism and survival in a damselfly: does host sex matter? *Proc. R. Soc. Lond. B.,* **268**: 1133-1137.

Crawley, M.J. 1992. *Natural Enemies.* Oxford: Blackwell Scientific Publications.

Corbet, P.S. 1999. *Dragonflies: Behavior and Ecology of Odonata*. Ithaca: Cornell University Press.

Decastaeker, E., De Meester, L. and Ebert, D. 2002. In deep trouble: habitat selection constrained by multiple enemies in zooplankton. *Proc. Nat. Acad. Sci. USA***, 99**: 5481-5485.

Eklöv, P. and Werner, E.E. 2000. Multiple predator effects on size-dependent behavior and mortality of two species of anuran larvae. *Oikos* **88**: 250-258.

Feder, D., Mello, C., Garcia, E. and Azambuja, P. 1997. Immune responses in *Rhodnius prolixus*: influence of nutrition and ecdysone. *J. Ins. Physiol.* **43**: 513-519.

Fellowes, M.D.E and Godfray H.C.J. 2000. The evolutionary ecology of resistance to parasitoids by *Drosophila*. *Heredity* **84**: 1-8.

Gillespie, J., Kanost, M. and Trenczek, T. 1997. Biological mediators of insect immunity. *Ann. Rev. Entomol.,* **42**: 611-643.

Goldsby, R.A., Kindt, T.J. and Osborne, B.A. 2000. *Kuby Immunology*. New York: Freeman.

Inden-Lohmar, C. 1997. Sukzession, Struktur und Dynamik von Libellenpopulationen an Kleingewässern, unter besonderer Berücksichtigung der Ökologie von *Aeshna cyanea* (Müller, 1764), PhD thesis, University of Bonn.

Johansson, F. 1996. The influence of cannibalism and prey density on growth in the damselfly *Coenagrion hastulatum*. *Arch. Hydrobiol.* **137**: 523-535.

Johansson F. and Leonardsson, K. 1998. Swimming speeds and activity levels of consumers at various resource and consumer densities under predation risk. *Can. J. Zool.,* **76**: 76-82.

Johansson, F. and Rowe, L. 1999. Life history and behavioral responses to time constraints in a damselfly. *Ecology* **80**: 1242-1252.

Koperski, P. 1997. Changes in feeding behaviour of the larvae of the damselfly *Enallagma cyathigerum* in response to stimuli from predators. *Ecol. Entomol.* **22**: 167-175.

Kurtz, J., Wiesner, A., Götz, P. and Sauer, K. 2000. Gender differences and individual variation in the immune system of the Scorpionfly *Panorpa vulgaris* (Insecta: Mecoptera). *Develop. Comp. Immunol.* **24**: 1-12.

Kurtz, J., Klappert, K. , Schneider, W. and Reinhold, K. 2002. Immune defence, dispersal and local adaptation *Evol. Ecol. Res.,* **4.** 431-439.

Lass, S. and Bittner, K. (2002) Facing multiple enemies: parasitised hosts respond to predator kairomones. *Oecologia* **132**. 344-349.

Leung, B., Baker, R. and Forbes, M. 1999. Grooming decisions by damselflies, age-specific colonisation by water mites, and the probability of successful parasitism. *Int. J. Parasitol.,* **29**: 397-402.

Marden, J. 1989. Bodybuilding dragonflies: costs and benefits of maximizing flight muscle. *Physiol. Zool.***62**: 505-521.

Mitchell, R. 1967. Host exploitation of two closely related water mites. *Evolution* **21**: 59-75.

Moore, J. 2002. *Parasites and the Behavior of Animals*. Oxford, Oxford University Press

Plaistow, S. and Siva-Jothy, M. T. 1996. Energetic constraints and male mate-securing tactics in the damselfly *Calopteryx splendens xanthostoma (*Charpentier). *R. Soc. Lond. B.,* **263**: 1233-1238.

Plaistow, S. and Siva-Jothy, M.T. 1999. The ontogenetic switch between odonate life history stages: effects on fitness when time and food are limited. *Anim. Behav.,* **58**: 659-667.

Rigby, M. and Jokela, J. 2000. Predator avoidance and immune defence: costs and trade-offs in snails. *R. Soc. Lond. B.,* **267**: 171-176.

Rolff, J. 1999. Parasitism increases offspring size in a damselfly: experimental evidence for parasite-mediated maternal effects. *Anim. Behav.,* **58**: 1105-1108.

Rolff, J. 2001. Evolutionary ecology of water mite-insect interactions: a critical appraisal. *Arch. Hydrobio.,* **152**: 353-368.

Rolff, J. 2002. Bateman's principle and immunity. *R. Soc. Lond. B.,* **269**: 867-872.

Rolff, J. and Joop, G. 2002. Estimating condition: pitfalls using weight as a fitness correlate. *Evol. Ecol.y Res.,* **4**: 931-935.

Rolff, J. and Siva-Jothy, M.T. 2003. Invertebrate Ecological Immunology. *Science* **301:** 472-475.

Ryder, J. and Siva-Jothy, M.T. 2001. Quantitative genetics of immune function and body size in the house cricket, *Acheta domesticus*. *J. Evol. Biol.* **14**: 646-653.

Schmid-Hempel, P. 2003. Variation in immune defence as a question of evolutionary ecology. *R. Soc. Lond. B.*, 270: 357-366.

Sih , A., Englund, G. and Wooster D. 1998. Emergent impacts of multiple predators on prey. *Trends in Ecol. Evol.,* **13**: 350-355.

Sokolovska, N., Rowe, L. and Johansson, F. 2000 Fitness and body size in mature odonates. *Ecol. Entomol.,* **25:** 239-248.

Stoks, R. 1999. The effect of lamellae autotomy and sexual size dimorphsim on startle-response performance in larvae of an lestid damselfly (Odonata). *J. Zool., Lond.,* **247**: 269-273.

Stoks, R., Block, M. d., Gossum, H. v., Valck, J., Lauwers, K., Verhagen, R., Matthysen, E. and Bruyn, L. d. 1999. Lethal and sublethal costs of autotomy and predator presence in damselfly larvae. *Oecologia* **120**: 87-91.

Stoks, R. and McPeek, M.A. 2003. Antipredator behaviour and physiology determine Lestes species turnover along the pond gradient. *Ecology*, **84:** 1576-1587.

Sugumaran, M. 2002. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigm. Cell Res.,* **15**: 2-9.

Suhling, F. and Lepkojus, S. 2001. Differences in growth and behaviour influence asymmetric predation among early-instar dragonfly larvae. *Can. J. Zool.,* **79**: 854-860.

Traniello, J., Rosengaus, R. and Savoie, K. 2002. The development of immunity in a social insect: evidence for the group facilitation of disease resistance. *Proc. Nat. Acad. Sci. USA,* **99**: 6838-6842.

Wilson, K., Cotter, S., Reeson, A. and Pell, J. 2001. Melanism and disease resistance in insects. *Ecol. Letters,* **4**: 637-649.

Worley, A., Houle, D. and Barrett, S. 2003.Consequences of hierarchical allocation for the evolution of life-history traits. *Am. Nat.,***161**: 153-167.

Zuk, M. and Stoehr, A. 2002. Immune defense and host life history. *Am. Nat.,* **160**: S9-S22.

Chapter III

Colour as a signal of parasite resistance according to the hypothesis by Hamilton and Zuk (1982) and Folstad and Karter (1992)?

Immune function and parasite resistance in male and polymorphic female *Coenagrion puella*

Abstract

Background: Colour polymorphisms are widespread and one of the prime examples is the colour polymorphism in female coenagrionid damselflies: one female morph resembles the male colour (andromorph) while one, or more, female morphs are described as typically female (gynomorph). However, the selective pressures leading to the evolution and maintenance of this polymorphism are not clear. Here, based on the hypothesis that colouration and especially black patterning can be related to resistance against pathogens, we investigated the differences in immune function and parasite resistance between the different female morphs and males.

Results: Our field and experimental infection data revealed no differences in immune function or resistance between the female morphs but between the sexes. Females, however, show higher resistance against a fungal pathogen. This pathogens invades through the cuticle and females have a much higher black content than males.

Conclusions: With respect to resistance and immune function ‘andromorph’ blue females of *Coenagrion puella* do not resemble the males. Therefore the colour polymorphism in coenagrionid damselflies is unlikely to be maintained by differences in immunity.

Introduction

Colour polymorphisms are widespread and prominent in e.g. coral reef fish [1], birds [2] and tree frogs [3]. In many cases the genetic basis has been investigated [1,4]. However, the nature of the selection pressures maintaining such polymorphisms are rarely known. One example where the selective pressure is well established for polymorphism is in the pea aphid *Acyrthosiphon pisum*. In this species, both red and green colour morphs occur; these morphs differ in their susceptibility against natural enemies: the green morph is more susceptible to parasitism while red morphs suffer from higher predation [5]. Polymorphisms can also be maintained by sexual selection, as suggested for plumage coloration in some birds [6] and in guppies [7].

Frequently, colour polymorphism is restricted to one sex. Female-limited colour polymorphism occurs for example in the lizard *Uta stansburiana*: females have either a yellow or an orange throat. These morphs follow different life history strategies: the orange morph pursues a r strategy, the yellow morph acts with a K-strategy. A trade-off between immunity and population density has been identified, such that the decline in antibody responsiveness with an increased number of neighbors is steeper in orange than in yellow females [8, 9]. Furthermore a genetic correlation between morphotype and immune responsiveness was reported [9]. Thus suggesting that this female colour polymorphism is maintained by the interaction between immune function and reproduction under fluctuating population densities.

In insects female colour polymorphisms are widespread among damselflies, such as polymorphism in female coenagrionids (Zygoptera, Odonata). Usually one female morph resembles the male, dubbed the andromorph, while one or more other “typical” female morphs are called gynomorphs [10]. This polymorphism has been

shown to be genetically determined in at least three coenagrionid species [11, 12, 13, 14] and is most likely to be the case in all coenagrionids. In the azure damselfly *Coenagrion puella*, an andromorph, a gynomorph and an intermediate female morph have been described [15, Joop et al. in review].

Several hypotheses to explain the evolution and maintenance of this polymorphism have been suggested. Broadly they fall into two categories: (i) the density dependent/male mimic hypothesis [10, 16] or (ii) the learned mate recognition hypothesis (LMR) [17 and references therein] or variations on them [e.g. 18]. There is however no consensus yet on what drives female morph frequencies and maintains the polymorphism. Svensson et al. [19] recently showed that frequency dependent selection maintains the polymorphism in the wild in *Ischnura elegans*. Coenagrionids have a non-territorial mating system with a high rate of male harassment. Copulations can last several hours and females are forced to mate several times, even though they could store enough sperm to fertilize their entire egg number from only one mating [20]. Both hypotheses revolve around this issue. The male mimic hypothesis states that andromorphs mimic males to avoid male harassment with the risk of no matings under low population densities [16]. In contrast, according to the LMR idea males learn to recognize the most common morph in the population, which usually is the andromorph [summarized in 21, but see 22] and therefore the less common morph can avoid harassment. This leads to the conclusion that, depending on population and environmental factors the different morphs have different mating frequencies.

Here we propose that female colour polymorphism could be related to immune function and that selection on immune function and melanism could contribute to the maintenance of the colour polymorphism. There are three independent findings that make this hypothesis interesting and pertinent to study. (a) *C. puella* shows a sex

difference in investment in immune function during the larval stage [23] and in resistance to parasites [24]. Females have a higher haemocyte load and a higher activity of the enzyme Phenoloxidase (PO), these are both important components of insect resistance [25]. Both of these immune defence traits have frequently been linked to higher resistance in insects [26, 27, 28]. (b) Female *C. puella* have larger black cuticular patterning (melanin) on their abdomens when compared to males, however there are no differences between the female morphs [Joop et al. in review]. The black pigment is melanin produced by the PO cascade [29]. The PO cascade is involved in the melanisation and sclerotization of the cuticle and the cuticle is an important first defense line of insects. This is particularly important for defence against fungal infections, as entomopathogenic fungi hydrolyze cuticular proteins [30, 29]. For example in the moth *Spodoptera littoralis* it was shown that melanic larvae are more resistant to entomopathogenic fungi [31]. The same finding was reported in adults of the mealworm beetle *Tenebrio molitor* [32]. (c) It has been reported that mating frequencies differ between the female morphs. In the damselfly *Matrona basilaris japonica* it was shown that mating reduced the ability of encapsulating antigens in a wild population [33]. Moreover, Rolff and Siva-Jothy [34] demonstrated in the mealworm beetle *Tenebrio molitor* that an important component of the immune system, the PO cascade, was down regulated in both sexes after mating, via a highly conserved hormonal pathway.

In this study we aim to elucidate the relationship between colour polymorphism and immune function in order to understand the maintenance of colour polymorphisms. Polymorphisms are supposedly costly on different levels in terms of a reduced fitness; e.g. maintaining the polymorphic system itself should be costly and depending on the polymorphism might lead to the production of intermorphs of lower fitness, or there could be some pleiotropic effects which again might limit the

response to selection and which should lead to a reduced fitness in that environment [35]. As discussed above, black patterning and immunity are closely linked in insects and therefore trade-offs between them are not unlikely; this is extended here by including colour. We expect the female morphs to differ from males and differences between the female morphs are also possible:

Specifically we investigated whether the female morphs and/ or the males differ in (i) immunity or (ii) resistance to a novel pathogen and (iii) whether there is any evidence for differential resistance in the field.

Methods

Study organism

Coenagrion puella (Zygoptera, Odonata) is a common and well studied species [e.g. 41, 23] of northern and central Europe, usually occurring in small ponds [56]. Larvae hibernate in later instars. Shortly before emergence the damselfly larvae move to shallow water regions, where water mites might settle onto them, while they only later attach to the adult and start parasitizing [57]. As damselflies keep on feeding after emergence they may become infested with endoparasites as well e.g. eugregarines, as has been shown for several other damselfly species [e.g. 53, 58, 59]. These endoparasites attach to the mid-gut epithelium, thus rupturing or blocking it which again can reduce longevity [60]. After adult emergence it is easy to distinguish gender but not the female colour morphs, as the individuals are not yet coloured. Becoming fully coloured takes, depending on weather conditions, seven to ten days. In this time the damselflies also reach sexual maturity. For *C. puella* three

female colour morphs are described [15, Joop et al. in review] an ‘andromorph’ (blue), a ‘gynomorph’ (green) and an ‘intermediate’ morph (bluegreen).

Immune parameters and condition

Damselflies were collected as adults from two different populations near Braunschweig, Lower Saxony, Germany, over the whole flight season 2003. Population 1 is a well established population of high density and older than 15 years while population 2 is not older than three years and is of lower density [Joop, pers. obs.]. The populations are far enough apart that they do not interbreed. We used only fully coloured and therefore sexually mature animals but made sure that they were not too old by checking their wings for damage [20]: those with damaged wings were excluded. Furthermore animals were checked for scars resulting from previously attached water mites If there were scars but no water mites the damselfly has mated at least once (*Arrenurus* water mites detach during oviposition, [57]) and they were excluded from the analysis.

Immune parameter

As immune parameters we measured PO activity (humoral immunity) and counted haemocytes (cellular immunity), for both we followed the protocol of Joop and Rolff [23]. To obtain the haemolymph, the damselfly’s thorax was perfused with 0.3 ml sodium cacodylate buffer (0.01M Na-Coc, 0.005M $CaCl_2$). 20 µl of the resulting solution was pipetted into one well of a multiwell slide (adhesive epoxy-coated 12-well slides, Roth L209.1) to estimate the number of haemocytes.

The rest of the haemolymph sample was frozen at –80°C to disrupt cell walls and stop enzyme function. To measure PO activity 40µl of the frozen haemolymph sample was defrosted and mixed with 100 µl distilled water, 20 µl phosphate buffered

saline (PBS) and 40 µl l-DOPA (4g/ml) as the substrate in 96 well cell culture plates. Samples were measured every 15 seconds over 30 minutes at 30°C and at a wavelength of 490 nm. The slope of the reaction was calculated using softmax pro software and V_{max}, the velocity of maximum substrate conversion, was recorded.

Condition

We measured dry weight and fat content as correlates of condition. Both have been shown to be good estimates of condition in *C. puella* [61]. As females store the eggs in their abdomen and therefore have a higher weight and fat content in this compartment, we only analysed the condition parameter for the thorax in order to have a better comparison between the sexes. Measurements were taken according to Joop and Rolff [23] and to correct for size, fatless weight was used.

Statistical analyses

As our estimates for immunocompetence and condition were highly correlated [see also 61] these data were analysed using a MANCOVA with fatless weight as covariate. Fatless weight is a better correction for size than headwidth and therefore should be used whenever possible. Number of haemocytes (square root transformed [62]), PO activity (ln-transformed [62]), dry weight and fat content were used as dependent variables, ‘colour morph’ (male; blue, green or blue-green females) and population (1 or 2) as factors. The MANCOVA was followed by tests of between-subjects effects and pairwise comparisons. All analyses were performed using SPSS 12.0.1.

Natural parasites

Water mites are known to be common ectoparasites of damselflies, of which *Arrenurus cuspidator* is the most common in *C. puella* at least in one of our study populations [52]. They only parasitise adult individuals but attach in the larval stage [57]. In order to get an accurate estimate of the abundance of parasitic water mites we counted the parasitizing water mites on the damselflies collected as above for the two different populations in 2003. In 2004 water mites were only counted for population 1.

Another common parasite in damselflies are gregarines [63], which parastize in the mid-gut. Gregarines have so far not been described for *C. puella*. Therefore we collected individuals of *C. puella* in the season 2004 and dissected their mid-gut [see 53]. In the dissected animals water mite numbers were also counted.

Statistical analyses

As the number of parasites is correlated to the host's size [52] for all individuals, headwidth was measured and included into analyses as a covariate. We found no correlation between the numbers of different parasites and therefore analysed them separately using ANCOVAs with number of parasites (square root transformed [62]) as the dependent variable and colour morph and population (2003 data only) as factors. All analyses were performed using SPSS 12.0.1.

Fungal infection experiment

Damselfly larvae were caught in the first two weeks in April 2004 in the area "Klei" near Braunschweig (Lower saxony, Germany, 52°21'N, 10°35'E), when they were in the final three instars. Larvae were randomly placed into one of eight tanks (length:width 36:30 cms, filled with 18 litres of de-chlorinated water). Fifty larvae were

placed in each tank. Gauze was provided as a climbing structure, allowing the larvae to leave the water for hatching. Tanks were a priori assigned to control or fungal treatment. Tanks were placed in a temperature controlled room at 16°C and a light-cycle of 12:12h. All damselflies were held under the same feeding conditions. Tanks were monitored every morning for hatching individuals, the first adult damselflies were found on 17th June 2005.

As a fungal pathogen we used *Metarrhizium anisopliae*, which is known to be an entomopathogenic fungus [64]. Spores of *M. anisopliae* attach to the insect's cuticle and grow through the cuticle. Several fungi are known to attack damselflies [65, 36], but *M. anisopliae* is not a natural pathogen of *C. puella*. We chose this novel pathogen because we were interested in resistance and not host-parasite co-evolution [see 31, 32].

Spore suspension of *M. anisopliae*

M. anisopliae (strain F142) was grown on PDA (Potato Dextrose Agar, Merck) and later on 2 % biomalt agar for better sporulation at 25°C in petri dishes. To obtain the hydrophobe spores a 0.05 % Triton X 100 suspension was pipetted onto the petri dish, spores wiped off and their concentration adjusted using a Thoma haemocytometer [32]. Spore concentrations were between 1.4x10^7 and 3.0x10^7 spores per ml. Spore suspensions were stored in the fridge for no longer than 24h before use [37].

Inoculation with *M. anisopliae*

Imaginally eclosed damselflies were collected every morning. Their head width as a measure of size [41] and the fresh weight were recorded. The abdomen of control individuals was dipped in microcentrifuge tubes each containing 1.5 ml Triton-

X-100, while treatment individuals were dipped in 1.5 ml spore suspension (in Triton-X-100). Adult damselflies were held in 500 ml plastic containers with wet filter paper to provide humidity. Containers were closed with gauze. To prevent cross-infection inoculated treatment damselflies were kept in a separate 1 m^3 glass cube. To have optimal starting conditions for the spores to grow, all animals were kept for 8h at 27°C [64]. Thereafter damselflies were kept at 16°C, which is the ideal temperature to keep them alive as long as possible without feeding [Joop, unpublished data]. Under bad weather conditions damselflies can survive in the wild without hunting for several days [20] so our experimental conditions are not unnatural in this respect. Furthermore under bad weather conditions, especially rainy but not too cold (above 10°C) weather fungal growth is higher [64] and therefore damselflies are most likely to struggle with fungal infections. Survivorship was recorded daily. Dead damselflies were collected and their surface sterilized using 70% Ethanol and sterile distilled water. [Draeger, pers. comm.]. These damselflies were subsequently put into a sterile petri dish with humid filter paper and checked after 2, 4 and 6 weeks for fungal growth and whether this was *M. anisopliae*. This was necessary to make sure that the fungal infection worked and to control the control individuals for fungal growth as well.

Statistical analyses

Survival was analysed using a Cox regression as implemented in R [66]. As all animals died our data are non-censored. Days till death was used as a time estimate and treatment (control, infected) and colour morph (male; blue, green or blue-green female) or sex (male, female) as co-variates. The performance of the models is compared using AIC [66].

Results

Immune parameters and condition

Table 1: MANCOVA (Pillai's trace) for immunity and condition for the different colour classes and populations in 2003, controlling for size (fatless weight).

source	value	F	Hypothesis df	Error df	p
colour class	0.147	3.870	9	675.00	< 0.001
population	0.127	10.783	3	223.00	< 0.001
colour class x population	0.095	2.457	9	675.00	0.009
fatless weight	0.972	2457.220	3	223.00	< 0.001

A MANCOVA (Table 1) revealed that colour classes (male; blue, intermediate and green female) as well as populations differ in immunity and condition.

Table 2: Tests of between subjects effects for immunity and condition for the different colour classes and populations in 2003

source	dependent variable	df	F	p
colour class	PO activity [ln]	3	7.535	< 0.001
	haemocytes [sqrt]	3	4.593	0.004
	dry weight	3	2.903	0.036
	fat content	3	2.903	0.036
population	PO activity [ln]	1	1.376	0.242
	haemocytes [sqrt]	1	3.226	0.074
	dry weight	1	28.106	< 0.001
	fat content	1	28.106	< 0.001
colour class x population	PO activity [ln]	3	3.048	0.030
	haemocytes [sqrt]	3	2.422	0.067
	dry weight	3	1.790	0.150
	fat content	3	1.790	0.150

The between subjects tests (Table 2) showed, that the populations differ only in condition but not in immune parameters, which might be explained by different food or predator abundance. All female morphs group together and differ from the males (Table 3), except for dry weight and fat content, where the intermediate females differ neither to the other female morphs nor to the males. Condition, PO activity, dry weight and fat content was always higher in females (Fig. 1).

Table 3: Pairwise comparisons for immunity and condition in the colour classes.

dependent variable	colour class (1)	colour class (2)	mean difference (1-2)	SE	p
PO activity [ln]	blue female	green female	0.579	0.0531	0.277
		intermediate female	0.265	0.0568	0.641
		male	1.860	0.0542	0.001
	green female	intermediate female	- 0.314	0.0395	0.428
		male	1.281	0.0364	0.001
	intermediate female	male	1.595	0.0405	< 0.001
haemocyte [sqrt]	blue female	green female	0.120	0.0431	0.781
		intermediate female	0.469	0.0462	0.311
		male	1.133	0.0441	0.011
	green female	intermediate female	0.348	0.0321	0.279
		male	1.013	0.0296	0.001
	intermediate female	male	0.665	0.0330	0.045
dry weight [mg]	blue female	green female	- 0.028	0.045	0.533
		intermediate female	-0.046	0.048	0.338
		male	- 0.106	0.046	0.022
	green female	intermediate female	- 0.018	0.034	0.590
		male	- 0.078	0.031	0.012
	intermediate female	male	-0.060	0.034	0.083
fat content [mg]	blue female	green female	- 0.028	0.045	0.533
		intermediate female	-0.046	0.048	0.338
		male	- 0.106	0.046	0.022
	green female	intermediate female	- 0.018	0.034	0.590
		male	- 0.078	0.031	0.012
	intermediate female	male	-0.060	0.034	0.083

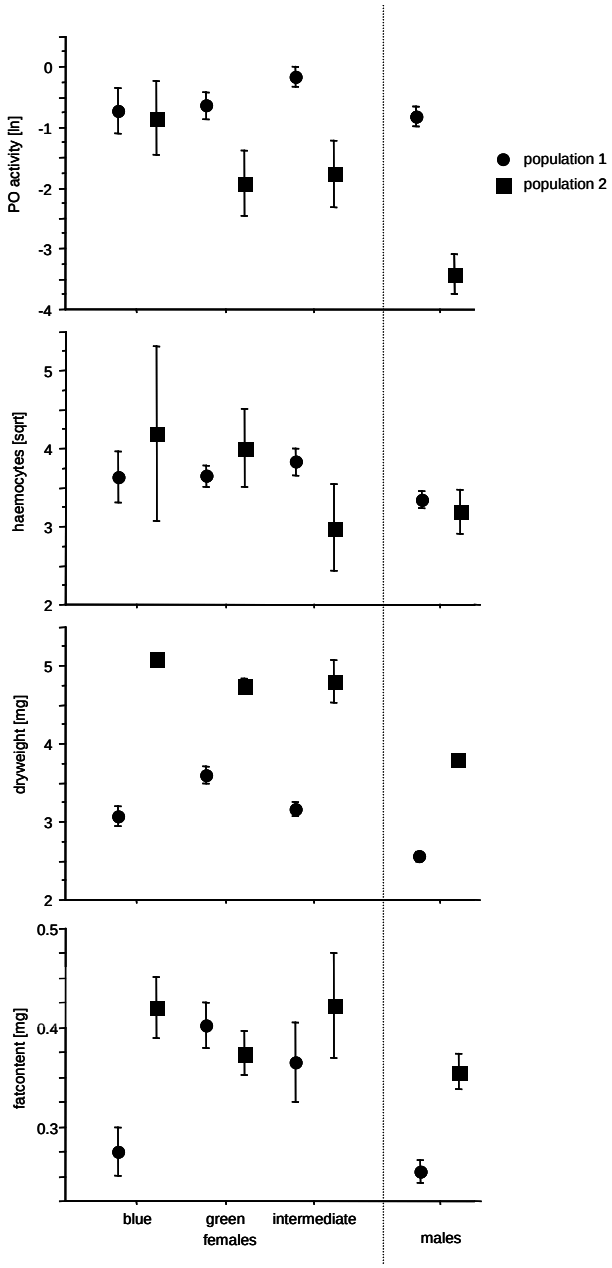

Figure 1: Means for immune parameters and condition for all the female morphs and the males of the two populations in 2003, SE is given.

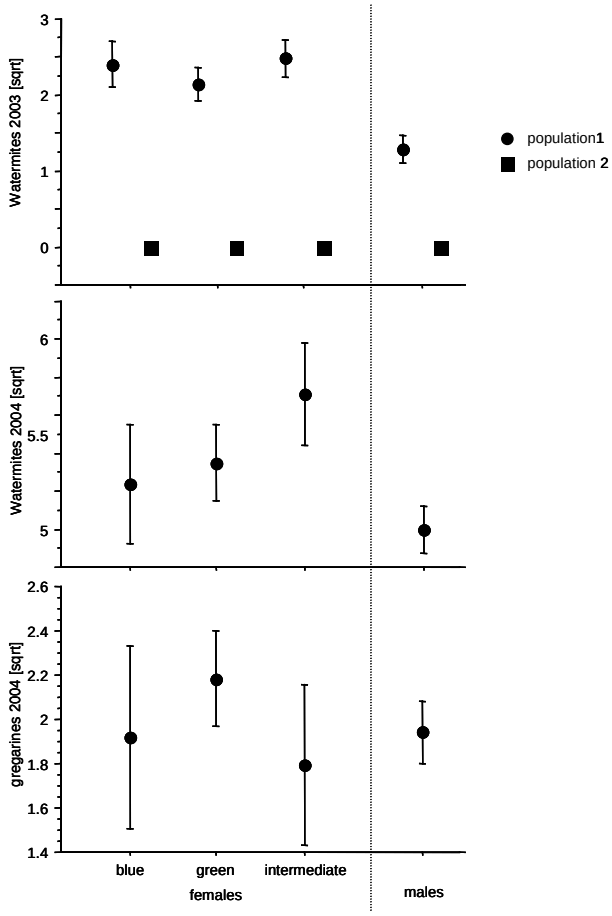

Figure 2: Means for natural parasite infection for the two populatio ns combined in 2003 and for population 1 in 2004 for the different morphs, SE is given.

Table 4: ANCOVAs for natural parasites in 2003 and 2004 for the different colour classes and populations (2003 only), controlling for size (head width). All parasite numbers were square root transformed.

source	dependent variable	df	F	p
colour class	watermites 2003	3	0.605	0.612
	watermites 2004	3	2.261	0.082
	gregarines 2004	3	0.419	0.740
population	watermites 2003	1	12.201	0.001
colour class x population	watermites 2003	3	1.886	0.682
head width	watermites 2003	1	8.397	0.004
	watermites 2004	1	11.428	0.001
	gregarines 2004	1	0.024	0.877

Natural parasites

We did not find a difference in water mite or gregarine abundance for the colour classes (Table 4). Water mite infestation differs between the populations in 2003 with hardly any water mites present in population 2. In general, infestation with these parasites seems not to be very high and with only slight differences for water mites between 2003 and 2004 (Fig. 2).

Fungal infection experiment

First we tested whether males and females differ in their survival, which is highly significant for sex (n=378, Z=-3.84, p=0.00012) and treatment (n=378, Z=-4.67, p<0.0001) (Fig. 3a), therefore fungal treatment reduced survival differently depending on sex, and females survived better. In a second analysis we tested for survival differences between the different female morphs (Fig. 3b). Again the fungal treatment has an influence on survival (n=159, Z=-2.263, p=0.024) but between the female morphs no significant differences were found (n=159, Z=-0,998, p=0.320). In a third model we used treatment and colour morph including males as covariates. Both covariates were significant (n= 378, colour morph: Z=-3.72, p=0.0002, treatment: Z= -4.53, p<0.0001). Subsequently we compared the first model (only gender differences) with the third model (that included female colour morphs and males). However, we did not find a difference in the performance of these models (Difference

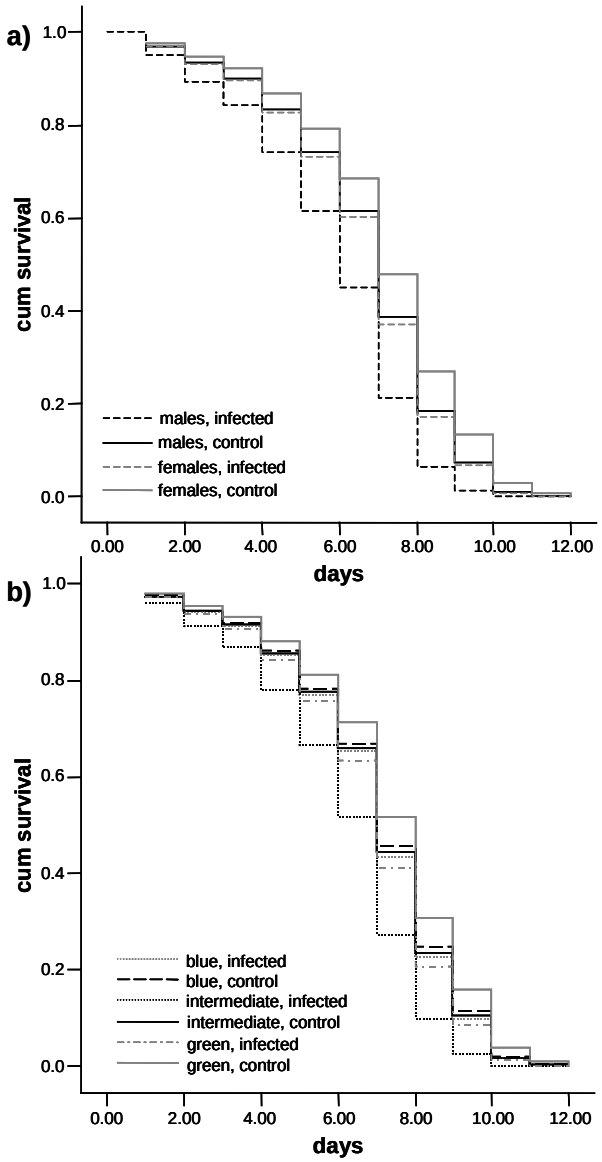

Figure 3: Survival function for the different morphs after fungal infection and for non-infected controls, a) comparing males and all females, b) comparing the different female morphs.

in AIC between model 1 and 3: as this is >2, the models do not differ). The differences in general colour morph in model 3 are explained by the differences between males and females alone.

Discussion

We found significant differences between males and females, with females having a stronger immune function as indicated by the higher haemocyte counts and PO activity. Moreover, females are more resistant against fungal infection with *M. anisoplae*. However, we did not find any such differences between the female morphs. We found no evidence for any differences in parasite abundance in the field, neither between the sexes nor within the female colour morphs. Why do females invest more in resistance and immunity? And why are there no differences between the female colour morphs?

Adult females have generally a higher PO activity and a higher haemocyte load than the adult males in *C. puella*. Therefore our results support the data of Joop and Rolff [23], where the sexes differed in their investment in immunity immediately

after emergence. This shows that the sexual dimorphism is maintained later in the adult life. The differences in immune function were also reflected in the higher resistance of females against the fungal pathogen. Female *C. puella* have a higher black melanin content in their cuticle than males (Joop et al. under review). Black patterning of insect cuticle is commonly a product of melanin, a pigment, that has antimicrobial properties and which is produced via the PO cascade [25]. *M. anisopliae* and most other entomopathogenic fungi of the order Cordyceps have spores that produce a lysozyme which dissolves the insect's cuticle [30, 36]. The spores can then enter the haemocoel and parasitise the host [37]. Wilson et al. [31] have shown that melanic moths exhibit a higher resistance towards entomopathogenic fungi than non-melanic individuals. Furthermore, Barnes and Siva-Jothy [32] found a positive correlation between cuticular melanisation and resistance to *M. anisopliae* in the mealworm beetle *Tenebrio molitor*. This might be explained by the fact that darker cuticle contains more melanin [38, 29]. All female morphs in *C. puella* have about twice as much black content than males [Joop et al., under review], this could be a major mechanism for their higher resistance towards the fungal treatment. Moreover, females invest more in PO, which is most likely involved in resistance against the fungal infection [29]. It could be speculated that in other coenagrionid damselfly species such as *Ischnura elegans*, where males and females are almost similar in black patterning, no such differences in fungal resistance occur. If this is true, this would constitute a potential cost of being a nearly perfect male mimic in *C. puella*: females would forfeit their higher resistance by decreasing their black melanin content.

The differences in immune function and resistance between the sexes can be explained by differences in life-history, males increase their fitness by increasing their mating rate while females increase their fitness by longevity [39, 40, 41, 42].

Furthermore females have a much higher investment in reproduction than males, as egg production is much more energy consuming than the production of sperm [43, 42 and references therein]. Because females achieve higher fitness through longevity and pay higher costs for reproduction, they should invest optimally in immunity as well to ensure a long reproductive life.

An additional selection pressure on black patterning may be found in thermoregulation. The mortality of mitosporic fungi such as *M. anisopliae* has been shown to be highly dependent on the environmental temperature and therefore the insect's body temperature [e.g. 44, 45]. Locusts use this and actively seek places with higher temperature after fungal infection in order to overcome it [46]. Male *C. puella* spend most of their time near the water waiting for females, while females spend more time searching for prey and resting in hedges [47, 48]. Therefore males are probably exposed to higher temperatures than females. The higher black content might help the female to gain higher body temperature while being exposed to the sun. Furthermore, it has been suggested that male *C. puella* are capable of thermoregulatory colour change [15]. This thermoregulation is assumed to protect flight muscles and sperm from overheating. It is unknown whether eggs also need to be protected from exposure to high temperatures. Therefore it could be, that female cuticle does not simply have a higher black melanin content, but that reduced melanin patterning in males is adaptive to avoid overheating. With the more complex structure of the female cuticle due to the higher melanin content, the cuticle might also give additional UV protection but certainly more data on this would be needed.

Blue females are dubbed andromorphs [10] and also are supposed to mimic male behaviour to avoid male harassment [10, 48, 50, 51], why did we find no differences in immune defence between the female morphs? As all females have the same reproductive requirements in terms of for example egg production and

oviposition, this might outweigh any behavioural differences between the morphs. Contrary to our expectations the supposed different mating tactics in the female morphs do not seem to mirror different immune strategies, as might be suggested by the findings of Rolff and Siva-Jothy [34] and was found in *U. stansburiana* [19]. Longevity should of course be the overriding factor, but it seems that this is not achieved by different strategies involving immunity and resistance.

From a broader perspective this is supported by our results for parasitism in the wild. We found no significant differences over all for water mite or gregarine load. Taking into account the fact that all damselfly larvae (both genders and all female morphs) hatch and move to more shallow areas of the pond inhabited by potentially infecting water mites, it is not surprising that we found no differences in parasite load between gender or morphs [52]. Gregarines are ingested with the food, either in the larval or adult stage. And as long as the food infected is equally distributed over the population's habitat and they eat the same food types, it is not surprising that no differences in parasite load were found. As a next step it should be studied whether the parasites differ in their effect on the female morph`s longevity: Braune and Rolff [24] demonstrated that the sexes differed in their survival, which was also dependent on water mite load. Furthermore females need to feed more because of the expenditure upon egg production. Therefore females have an increased risk of gregarine infection. However in other damselfly species it has been shown that PO activity is positively correlated with resistance against gregarines [53] and that gut and haemolymph PO activity is positively correlated [54]. Even though we have no data on this link in *C. puella*, one possible explanation is that females have a higher gregarine infection risk, yet because of their higher investment in immunity this is not reflected in the abundance of gregarines in hosts in the wild.

Conclusion

In conclusion, our results do not support the idea that blue females of *C. puella* are male like with respect to immunity and resistance. This is probably due to the selection pressure upon female morphs to have the same reproductive level. It is probable that the female morphs have different costs associated with maintaining this standard level of reproduction and therefore have to trade-off elsewhere, e.g. in clutch- or egg size [55], especially as reproduction seems to be of overriding importance.

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References

1. Munday PL, Eyre PJ, Jones GP: **Ecological mechanisms for coexistence of colour polymorphism in a coral-reef fish: an experimental evaluation.** *Oecologia* 2003, **442**: 519-526.

2. Galeotti P, Rubolini D, Dunn PO, Fasola M: **Colour polymorphism in birds: causes and functions.** *J Evol Biol* 2003, **16**: 635-646.

3. Wente WH, Phillips JB: **Fixed green and brown color morphs and a novel color-changing morph of the pacific tree frog *Hyla regilla*.** *Am Nat* 2003, **162**: 461-473.

4. Quigley IK, Turner JM, Nuckels RJ, Manuel JL, Budi EH, MacDonald EL, Parichy DM: **Pigment pattern evolution by differential deployment of neural crest and post-embryonic melanophore lineages in *Danio* fishes.** *Development* 2004, **131**: 6053-6069.

5. Losey JE, Ives AR, Harmon J, Ballantyne F, Brown C: **A polymorphism maintained by opposite patterns of parasitism and predation.** *Nature* 1997, **388**: 269-272.

6. Andersson M: *Sexual Selection*. - Princeton University Press; 1994.

7. Kodric-Brown A: **Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*).** *Behav Ecol Sociobiol* 1985, **17**: 199-205.

8. Sinervo B, Bleay C, Adamopoulou C: **Social causes of correlational selection and the resolution of a heritable throat color polymorphism in a lizard.** *Evolution* 2001, **55**: 2040-2052.

9. Svensson EI, Abbott J, Härdling R: **Female polymorphism, frequency dependence, and rapid evolutionary dynamics in natural populations.** *Am Nat* 2005, **165**: 567-576.

10. Robertson HM: **Female dimorphism and mating behaviour in a damselfly, *Ischnura ramburi*: females mimicking males.** *Anim Behav* 1985, **33**: 805-809.

11. Johnson C: **The inheritance of female dimorphism in the damselfly, *Ischnura damula*.** *Genetics* 1964, **49**: 513-519.

12. Johnson C: **Genetics of female dimorphism in *Ischnura demorsa*.** *Heredity* 1966, **21**: 453-459.

13. Cordero A: **The inheritance of female polymorphism in the damselfly *Ischnura graellsii* (Rambur) (Odonata: Coenagrionidae).** *Heredity* 1990, **64**: 341-346.

14. Andres JA, Cordero A: **The inheritance of female colour morphs in the damselfly *Ceriagrion tenellum* (Odonata, Coenagrionidae).** *Heredity* 1999, **82**: 328-335.

15. Sternberg K: ***Coenagrion puella***. In: *Die Libellen Badenwürttembergs, Kleinlibellen (Zygoptera). E*dited by Sternberg K, Buchwald L. Stuttgart: Verlag Eugen Ulmer; 1999: 278-287.

16. Hinnekint BON: **Population dynamics of *Ischnura E. elegans* (Vander Linden) (Insecta:Odonata) with special reference to morphological colour change, female polymorphism, multiannual cicles and their influence on behaviour.** *Hydrobiologia* 1987, **146**: 3-31.

17. Fincke OM: **Polymorphic signals of harassed odonates and the males that learn them support a novel frequency-dependent model**. *Anim Behav* 2004, **67**: 833-845.

18. Sherratt TN: The evolution of female-limited polymorphisms in damselflies: a signal detection model. *Ecolo. letters* 2001, **4**: 22-29.

19. Svensson E, Sinervo B, Comendant T: **Density-dependent competition and selection on immune function in genetic lizard morphs.** *Proc Nat Acad Sci USA* 2001, **98**: 12561-12565.

20. Corbet PS: *Dragonflies - behaviour and ecology of Odonata*. Harley Books; 1999.

21. Miller MN, Fincke OM: **Cues for mate recognition and the effect of prior experiences on mate recognition in *Enallagma* damselflies.** *Journ. of Ins. Behav.* 1999*,* **12**: 801-814.

22. Forbes M: . **Test of hypotheses for female-limited polymorphism in the damselfly, *Enallagma boreale* Selys.** *Anim Behav* 1994, **47**: 724-726.

23. Joop G, Rolff J: **Plasticity of immunefunction and condition under the risk of predation and parasitism.** *Evol Ecol Research* 2004, **6**: 1051-1062.

24. Braune P, Rolff J: **Parasitism and survival in a damselfly: does host sex matter?** *Proc R Soc Lond B* 2001, **268**: 1133-1137.

25. Sugumaran M: **Comparative Biochemistry of Eumelanogenesis and the Protective Roles of Phenoloxidase and Melanin in Insects.** *Pigment and Cell Research* 2002, *15*: 2-9.

26. Braun A, Hoffmann J, Meister M: **Analysis of the Drosophila host defense in domino mutant larvae, which are devoid of hemocytes.** *Proc Nat Acad Sci USA* 1998, **95**: 14337-14342.

27. Siva-Jothy MT, Moret Y, Rolff J**: Evolutionary ecology of insect immunity. Invited review.** *Advanc Ins Physiol* 2004: in press.

28. Schmid-Hempel P: **Evolutionary Ecology of insect immune defence.** *Annu Rev Entomol* 2005, **50**: 529-551.

29. Gillespie J, Kanost M, Trenczek T: **Biological mediators of insect immunity.** *Annu Rev Entomol* 1997, **42**: 611-643.

30. Marmaras V, Charalambidis N, Zervas C: **Immune Response in Insects: The role of Phenoloxidase in Defence Reaktions in Relation to Melanisation and Sclerotisation.** *Arch Ins Biochem Physiol* 1996, **31**: 119-133.

31. Wilson K, Cotter SC, Reeson AF, Pell JK: **Melanism and disease resistance in insects.** *Ecol letters* 2001, **4**: 637-649.

32. Barnes AI, Siva-Jothy MT: **Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity.** - *Proc R Soc Lond B* 2000, *267*: 177-182.

33. Siva-Jothy MT, Tsubaki Y, Hooper R: **Decreased immune response as a proximate cost of copulation and oviposition in a damselfly.** *Physiol Entomol* 1998, **23**: 274-277.

34. Rolff J, Siva-Jothy MT: Copulation corrupts immunity: **A mechanism for a cost of mating in insects.** *Proc Nat Acad Sci USA* 2002, *99*: 9916-9918.

35. Moran N: **The Evolutionary maintenance of alternative phenotypes.** *Am Nat* 1992, **139**: 971-989.

36. Sung JM: ***Cordyceps* Diversity and its preservation in Korea.** *Inoculum* 2004, **55**: 1-3.

37. Goettel MS, Inglis GD: Fungy: Hyphomycetes. In: *Manual Techniques in Insect Pathology* (ed. Lacey, L.), 213-249. San Diego: Academic Press, 1999

38. Ashida M, Brey P: **Role of the integument in insect defence: Pro-phenoloxidase cascade in the cuticular matrix.** *Proc Nat Acad Sci USA* 1995, **92**: 10698-10702.

39. Bateman AJ: **Intra-sexual selection in *Drosophila*.** *Heredity* 1948, **2**: 349-368.

40. Banks M, Thompson D: **Lifetime mating success in the damselfly *Coenagrion puella*.** *Anim Behav* 1985, **33**: 1175-1183.

41. Banks M, Thompson D**: Lifetime reproductive success of females of the damselfly *Coenagrion puella*.** *J Anim Ecol* 1987, **56**: 815-832.

42. Rolff J: **Bateman's principle and immunity.** *Proc R Soc Lond B* 2002, **269**: 867-872.

43. Williams G: *Adapatation and Natural Selection*. - Princeton Univ. Press; 1996.

44. Stacey DA, Fellowes MDE: **Influence of temperature on pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae) resistance to natural enemy attack.** - *Bull Entomol Res* 2004, **92**: 351-357.

45. Thomas MB, Blanford S: **Thermal biology in insect-parasite interactions.** *Trends Ecol Evol* 2003, **18**: 344-350.

46. Quedraogo RM, Goettel MS, Brodeur J: **Behavioural thermoregulation in the migratory locust: a therapy to overcome fungal infection.** *Oecologia* 2004, **138**: 312-319.

47. Anholt B, Vorburger C, Knaus P: **Mark-recapture estimates of daily survival rates of two damselflies (*Coenagrion puella* and *Ischnura elegans*).** *Can J Zool* 2001, **79**: 895-899.

48. Cordero A: **Reproductive behaviour of *Ischnura graellsii* (Rambur) (Zygoptera: Coenagrionidae).** *Odonatologica* 1989, **18**: 237-244.

50. Van Gossum H, Stoks R, De Bruyn L: **Frequency-dependent male mate harassment in intra-specific variation in its avoidance by females of the damselfly *Ischnura elegans*.** *Behav Ecol Sociobiol* 2001, **51**: 69-75.

51. Sirot LK, Brockmann HJ, Marinis C, Muschett G: **Maintenance of female-limited polymorphism in *Ischnura ramburi* (Zygoptera: Coenagrionidae).** *Anim Behav* 2003, **66**: 763-775.

52. Rolff J: **Water mite parasitism in damselflies during emergence: two hosts, one pattern.** *Ecography* 2000*,* **23**: 273-282.

53. Siva-Jothy MT, Plaistow S: **A fitness cost of eugregarin parasitism in a damselfly.** *Ecolo Entomol* 1999, **24**: 465-470.

54. Siva-Jothy MT, Tsubaki Y, Hooper RE, Plaistow SJ: **Investment in immune function under chronic and acute challenge in an insect.** *Physiol Entomol* 2001, **26**: 1-5.

55. Rolff J, Schroeder B: **Regaining the water: a simulation model approach for Arrenurus larvae (Hydrachnella) parasitizing damselflies (*Coenagrion puella*: Odonata).** *Ecol Evol Acari* 1999*,* 359-366.

56. Askew RR: *The dragonflies of Europe.* Harley Books; 2004.

57. Rolff J: **Evolutionary Ecology of water mite - insect interactions: a critical appraisal.** *Arch Hydrobiol* 2001, **152**: 353-368.

58. Hecker KR, Forbes MR, Leonard NJ: **Parasitism of damselflies (*Enallagma boreale*) by gregarines: sex biases and relations to adult survivorship.** *Can J Zool* 2002, **30**: 162-168.

59. Tsubaki Y, Hooper RE: **Effects of Eugregarine parasites on adult longevity in the polymorphic damselfly *Mnais costalis* Selys.** *Ecol Entomol* 2004, **29**: 361-366.

60. Abro A: **The impact of parasites on adult populations of zygoptera.** *Odonatologica* 1990, **19**: 223-233.

61. Rolff J, Joop G: **Estimating condition: pitfalls of using weight as a fitness correlate.** *Evol Ecol Research* 2002, **4**: 931-935.

62. Sokal R, Rohlf F: *Biometry*. - W.H. Freeman and Company; 1995.

63. Abro A: **The mode of Gregarine infection in Zygoptera (Odonata).** *Zool. Scripta* 1976, **5**: 265-275.

64. Domsch KH, Gams W, Anderson TH: *Compendium of soil fungi*. - Academic Press; 1980.

65. Czeczuga B, Godlewska A, Mrozek E: **Zoosporic fungi growing in dead dragonflies (Odonata).** *Int Journ Odonatol* 1999, 2, 187-197.

66. Crawley MJ: *Statistical Computing. An introduction to data analysis using S-Plus*. John Wiley and Sons; 2002.

Chapter IV

Bateman's principle – '... the female is limited by egg production ...'

(Bateman 1948)

Clutch size, egg-size and -shape, and wing morphometry – how different are the female colour morphs in *Coenagrion puella*?

Abstract

The maintenance of female colour polymorphism in coenagrionid damselflies is still an open issue. Here we ask if the different female morphs of *Coenagrion puella* follow different reproductive strategies. We examined clutch size and egg morphometry of the morphs. Furthermore, as differences in clutch- or egg size might lead to different wing loads we measured wing morphometry for the female morphs and males. We found that blue females have smaller wings than gynomorphic females but do not differ in clutch- or egg size and therefore probably do differ in wing load and flight performance from the other female morphs. We also found that the female morphs differ in egg shape.

Introduction

The maintenance of colour polymorphism in damselflies is still unresolved. In gender specific polymorphism most work has been done on males so far: In demoiselle damselflies males can show more or less wing patterning and it has been shown that, depending on this males are either territorial or sneakers and therefore follow different mating strategies (Tsubaki et al. 1997, reviewed in Rüppell et al. 2005). However, only recently different female colour morphs have been linked to different reproductive strategies. For example in the side-blotched lizard *Uta stansburiana*, different reproductive strategies have been shown for the two female morphs (Svensson 2001). One acts as a K-strategist while the other acts as a r-strategist. In Drosophila it appears that red eyed females are male protected while brown eyed ones are not (Rice pers. comm.), which again implies different mating strategies.

In coenagrionid damselflies female colour polymorphism is common. Usually one female morph is coloured and sometimes also shows the same black patterning as the male, while at least one female morph differs in colouration (and patterning) from the male. For five species it has been shown that this polymorphism is genetically determined (Johnson 1964, Johnson 1966, Cordero 1990, Andres and Cordero 1999, Sanchez-Guillen et al. 2005), in *Ischnura elegans* over three alleles with a directional dominance. Andres et al. (2000) could also show that there is selection on the morphs in the wild but it is not known what it is acting on. Several options have been discussed: frequency or density dependence (Cordero 1989, Andres et al. 2000, Sherratt 2001), which seem to be important indeed (Svensson et al. 2005); behavioural differences (Gorb 1998); and differences in mating (e.g. Sirot and Brockmann 2001). All this is mainly discussed under the umbrella of the male

mimic hypothesis (Johnson 1966, Robertson 1985, Hinnekint 1987), which states that the male like female mimics the male to avoid male harassment. Male harassment is high in most coenagrionid species, as the sexes have different strategies to optimize fitness (Corbet 1999). Males should mate as many females as possible while for females one mating is enough to fertilize their eggs (Banks and Thompson 1985) and more matings increase the risk of predation (Zeiss et al. 1997). This might lead to sexual conflict. Furthermore, Abbott and Svensson (2005) found for the trimorphic *Ischnura elegans* that the maternal colour morph is correlated to the offspring larval development time. This is true for female and male offspring, hence they predict sexual conflict between selection for protandry and emergence times associated with the maternal morph. To resolve this potential sexual conflict the evolution of different morphs could be an alternative to speciation and species separation.

It has been shown for dragonflies, that they can follow different reproductive strategies and that these strategies are linked to the guarding obtained by the male during oviposition (Schenk et al. 2004). Depending on the reproductive strategy, female morphs could vary in clutch size or one morph might have a variable clutch size while the other does not. Furthermore females might differ in egg size, either in general or again by varying within or between clutches, or both variations, in clutch- and egg size might be combined. Egg size again has been shown to be correlated to larval size and development in odonates (Schenk et al. 2004 and references therein), probably linked by vitellin content, which is higher in bigger eggs (Nicolaro and Bradley 1980, Takesue et al. 1983, Bradley and Estridge 1997). Therefore egg size differences between the morphs are almost certainly good indicators for the existence of different oviposition tactics.

Thompson (1989) found no differences between the female morphs in *C. puella* in lifetime reproductive successes, measured as number of clutches laid.

However there are indications for different reproductive strategies: We observed male-like *Coenagrion puella* ovipositing alone (Gillen, unpublished), while this species usually oviposits in tandem (Sternberg 1999). An other coenagrionid species with female colour polymorphism, *I. elegans* is believed to oviposit always non-guarded (Sternberg 1999). Furthermore it is shown for *I. elegans* not only that its three morphs appear in different frequencies within a population, but also that different percentages per morph can be found in copula (Svensson et al. 2005). If there is anything like different reproductive strategies between the morphs, this again could explain the findings by Svensson et al. (2005) for *Ischnura elegans*, stating over 4 years that the populations under observance undergo cycles. They discuss these cycles to be frequency dependent, driven by sexual conflict. Morph fecundity seems to be negatively affected by frequency- and density-dependent male mating harassment.

As some of the data suggested above might be hard to obtain it could be useful having a correlate for this: Assuming that one morph produces bigger eggs than the other but has the same clutch size, this should lead to a higher weight. A higher weight again results in more energy being needed for takeoff. This can be achieved either by higher efforts during takeoff itself and maximizing flight muscle (Marden 1989) or by different wing shape. Different wing shapes result in different lift production (Marden 1987). Further more different shapes should allow different maneuvering (Wootton 1992). Therefore differences in wing morphometry would be consistent with the existence of different female strategies.

Here we ask the question whether female morphs in *C. puella* differ in clutch size, egg size and egg shape. Furthermore we examine the wing loading of the different morphs, and if the morph with the higher weight per clutch has bigger wings

to balance flight performance. The measurement of these traits will enable us to unravel differences between the morphs in reproductive strategies.

Materials and methods

Coenagrion puella is a common species in central Europe. As in many other coenagrionid species females are described to be polymorphic in colour while males are always blue. The female morphs are termed as andromorph, gynomorph or intermediate (Sternberg 1999). Usually gynomorphs show higher numbers within a population than any of the other female morphs (but see Forbes 1994). In the following we use the term colour class with the four options male, andromorph, gynomorph and intermediate female, if referring to both gender including all female morphs. *C. puella* oviposits in tandem with the male holding the female on her neck while she lays the egg in floating plants and reed. Eggs hatch before winter and larvae hibernate in the +/- F-4 instar. First larvae hatch into the adult stage from early May on and *C. puella* is on the wing till mid or late August (Askew 2004).

All animals were caught in the area Klei near Braunschweig, Germany. Data for wing morphometry are from 2005. Head width, which is a common measurements for size in odonates (Benke 1970) was obtained using a stereomicroscope. As an additional measure for size we took abdomen length, using a caliper. Wings were measured in more detail (wing length, - height and area) using an image analyzer (Optimas).

Data for clutch size of the different female colour morphs are from 2003 and 2004, while data for egg size are from 2004 only. For getting the eggs females were caught out of copula in the wild and brought into the lab. Svensson et al. (2005) could

show for *I. elegans* that female age has no influence on clutch size, even though we only collected clutches of mature females. For oviposition each female was put in a separate plastic box (20x8 cm, 5 cm in height) which contained wet filter paper over a wooden stick as oviposition substrate. Boxes were covered with gauze so females could not escape. After 48h females were taken out of the box and freed. Eggs were counted on a light table and in 2004 about 30 eggs, if that many were present, of each female were preserved in 80% EtOH and stored until further measurements. Before measuring egg length, -width and area, eggs were re-hydrated in 4% Formalin in distilled water for 48h, at 4°C to avoid mould. All egg size measurements were taken using an image analyzer (Optimas). Subsequently all eggs from the size measurements of each female were dried on a pre-weighted foil at 60°C over night. After cooling them down to room temperature the eggs were weighted on the foil and the average dry weight per egg per female calculated. As coenagrionids oviposit into substrate only it is not possible to say which eggs were laid first, therefore for each female the average egg length, - width, - area and – dry weight was calculated and used in the following analyses. Egg shape was calculated as the ratio of egg length to egg width for each female, using the individuals average egg length and –width.

Statistics

All analyses were done using SPSS 12.0.1.

Wing measurements

As all wing measures and the controlling measures, head width, abdomen length and day (day 1 = 18th May 2005) were highly correlated (all $p>0.05$) data were reduced using a principle component analyses (PCA). The resulting new non-correlated variables were analyzed separately in an ANOVA. Pair wise comparisons

based on estimated marginal means followed for the colour classes, whenever the ANOVA was significant.

Clutch size and egg size, egg shape

Clutch size was analyzed using an ANOVA with colour class (andromorph, gynomorph or intermediate) and year (2003 or 2004) as independent variables and egg number as dependent variable. Egg size was analyzed in a MANOVA with egg length, - width, -area and egg dry weight as dependent variables and colour class as independent variable. The ratio of average egg length to egg width was used as dependent variable in an ANOVA. The closer the ratio is to 1 the more spherical the egg is.

Results

Wing measurements

The PCA yielded three composite variables that are statistically independent, with eigenvalues greater than one. Together they explain 83.86% of the variance. The new variable pc1 is dominated by the wing morphometry data (loadings > 0.8), while on pc2 the size measurements load high (> 0.8) and on pc3 day is the object with the highest load. Subsequent ANOVAs revealed, that pc1 and pc2 differ significantly for colour class while pc3 does not (table 1).

Table 1: ANOVA results for wing measurement data with pc1, pc2, and pc 3 as dependent variables and colour class (blue, intermediate, green females; males) as independent variable.

source		Type III sum of squares	df	Mean sum of squares	F	p
colour class	**pc1**	68.048	3	22.683	61.560	<0.001
	pc2	25.902	3	8.634	11.103	<0.001
	pc3	4.479	3	1.493	1.515	0.215

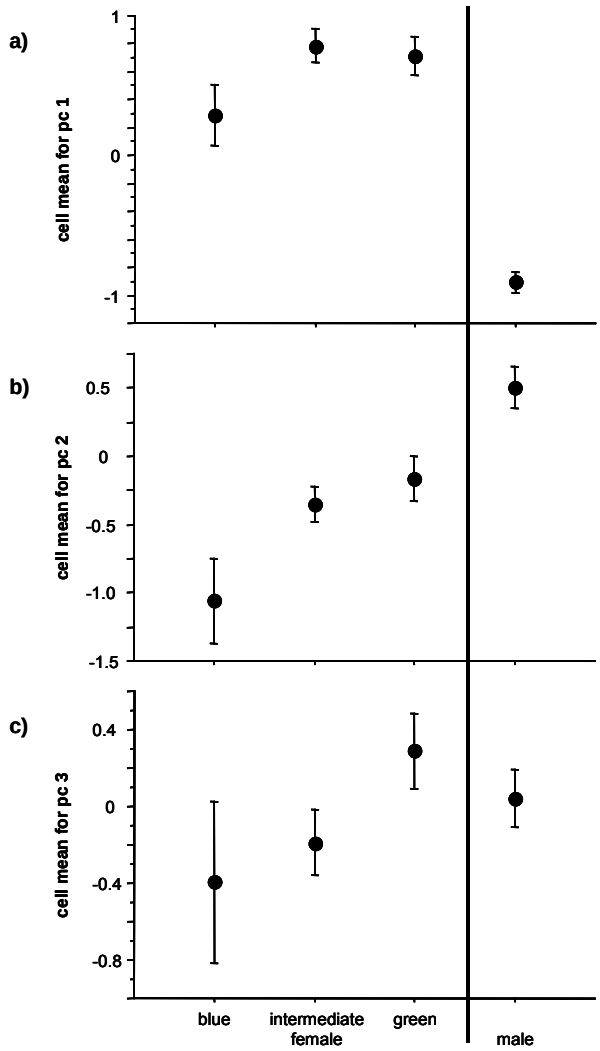

Figure 1: Cell mean for the colour classes (blue, intermediate and green female; male) for a) pc1, with wind data loading high; b) pc2, size data loading high; and c) pc3, day of emergence dominating. SE is given.

Therefore pairwise comparisons were conducted for pc1 and pc2 only. For both it was found, that blue females differ from the other females and males ($p<0.08$), males differ from all females ($p\leq0.03$). The patterns of pc1 and pc2 are different (figure 1): In wing morphometry (pc1) blue females are between the other females and males, contrasting in size measures (pc2) blue have the lowest cell mean, followed by intermediate and green females, males have the highest cell mean.

Clutch- and egg size, egg shape

The ANOVA for clutch size (figure 2) revealed no differences between the female morphs ($df=2$, $F=0.163$, $p=0.850$). Year also had no influence on clutch size ($SS=16842.317$, $df=1$, $MS=16842.317$, $F=1.050$, $p=0.307$). The MANOVA using egg width, length, area, and weight as dependent variables also revealed no significant differences between the female morphs (Pillai's trace: $F=1.462$, Hypothesis $df=10$, Error $df=106$, $p=0.164$) (figure3). Even though we found no significant differences for clutch- and egg-size between the female morphs, it is obvious that egg length, -area and –weight follow the same pattern for all morphs (figure 3). This might not appear significant, as variance especially in blue females is high. Contrasting for egg width the morphs show a different pattern which should lead to a more spherical three

dimensional shape in eggs of blue females, while intermediate females should have the most oval eggs: The ANOVA for egg shape showes that the morphs differ (df=2, F=4.837, p=0.0115) (figure 4). In a Fisher's PLSD test eggs from blue females differ significantly from those from intermediate females (p=0.0074), as do eggs from green females (p=0.0204). The differences in shape between eggs from blue and green females is not significant though (p=0.0930).

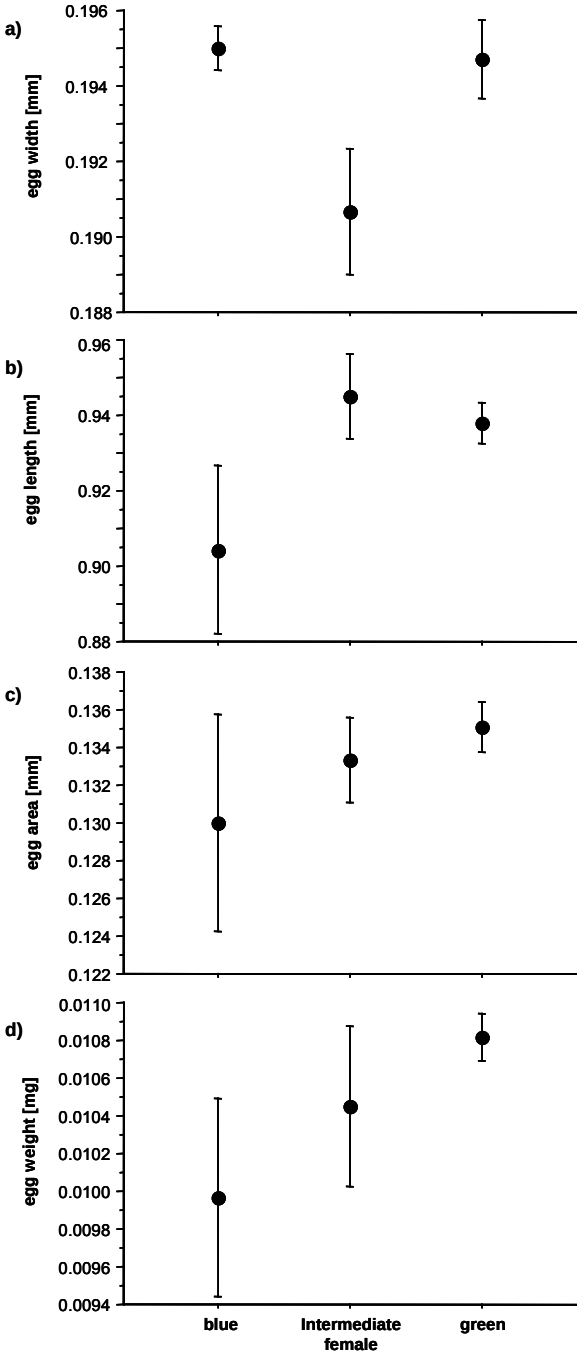

Figure 3: Mean egg data for the different female morphs in *C. puella*, a) egg width; b) egg length; c) egg area; and d) egg dry weight. SE is given.

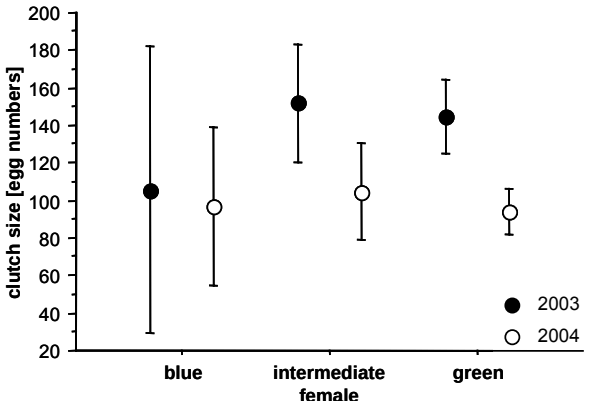

Figure 2: Mean clutch size for the different female morphs in *C. puella* for two different years. SE is given.

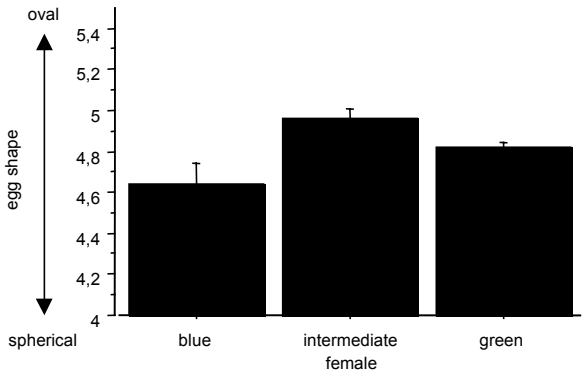

Figure 4: Egg shape, calculated as ratio of egg length to egg width is presented for the different female colour morphs in C. puella. SE is given.

Discussion

Our results revealed differences between the colour classes in wing measurement data including size but no differences in clutch- or egg size. In wing size blue females are between males and the other females and therefore closer to the males whom they are supposed to mimic (e.g. Hinnekint 1987)). However, in body size blue females are the smallest, while males are the biggest with the other female morphs grouping between them. All egg parameters besides egg width follow the same pattern, which results in different egg shapes for the different morphs. In the following we discuss possible explanations for the differences in wing morphometry and size, as these probably are not based on different reproductive strategies as was hypothesized before. However, we also discuss the idea of the different female morphs preferring different oviposition substrate, depending on egg shape.

Differences in adult size (pc 2) should result in weight differences, as size and dry weight are correlated in *C. puella* (Rolff and Joop 2002). Differences in weight were found between gender only (Joop et al. unpublished) when examining thorax weight. However this says nothing about the weight of the abdomen, that should be dominated by the egg production, as e.g. abdomen length and clutch size are positively correlated (Wigglesworth 1959). For egg weight no differences were found between the morphs, but this not necessarily stands for no abdominal differences: Over time one morph might produce generally more eggs, or more frequently and store them in the ovaries, even though Thompson (1989) could detect no differences in clutch number. And even though the vitellin content should be linked to egg size (Nocolaro and Bradley 1980, Takesue et al. 1983, Bradley and Estridge 1997) differences in development, as slight differences in diapause can not be excluded.

Unfortunately it was not possible to raise eggs of blue female *C. puella* in the lab while we had no problems with eggs of green or intermediate females, which in it self indicate differences in egg viability under these conditins. Even though no differences in clutch size were found this and other differences, as the frequency of egg production could be hard to detect.

Wing morphometry might provide an answer: As blue females are shown to have smaller wings than the other female morphs and are also smaller in size, it would be not surprising for blue females also to have smaller clutches and/or to produce smaller or lighter eggs to have a similar wing load as the other females. Wing load is defined as body weight per wing area (see Rüppell 1989) and is believed to have, besides wing shape and –mobility, high influence on flight performance. According to this blue females might be smaller than the other morphs but similar in wing load and therefore in flight performance. Contrary andromorph females are believed to mimic males. Males are known to be not as heavy as females (Joop et al. immunity paper) and have shorter wings, which should result in a different wing load. We could show that blue females even though being smaller than the other female morphs do not have smaller clutches or smaller/lighter eggs, but do have shorter wings. This might result in a higher wing loading in blue females which again could result in a flight performance more similar to males, at least different to the other female morphs. Therefore flight performance and maneuvering need to be observed more closely in the wild. At the moment most behavioural observations in coenagrionid damselflies did use tethered individuals (e.g. Gorb 1998), which probably does influence flight and therefore can not reveal, whether blue females perform more male like. Additionally analyses of flight performance under varying wind conditions might be helpful to reveal possible differences.

From the results discussed so far we can conclude that contrary to our hypothesis we could not reveal different reproductive strategies, in terms of clutch- or egg size for the female morphs, we found no differences in clutch- or egg size. Discussed differences in wing load might be a hint though. However, if it is not different reproductive strategies in the closer sense, what else maintains the female colour morphs in *C. puella*?

Surprisingly we found indications for the use of different oviposition substrates in the female morphs. Intermediate and green females have more elongated eggs, while blue females have more spherical eggs. *C. puella* oviposits in floating water plants and reed (Sternberg 1999). To our knowledge it has not been observed that one female morph prefers one or the other substrate. Females pierce the plant with their ovipositor and inject their eggs (Corbet 1999). However, injecting eggs should be easier with oval eggs than with spherical ones in harder substrates. On the other hand in soft material oval eggs are more likely to drop out of the plant again. Therefore blue females might prefer softer oviposition substrate while the other morphs oviposit in harder substrates. This is also supported by the different wing loads discussed above, different maneuvering might be helpful to reach one or the other substrate. If males were aware of this differences they might choose one morph or the other, depending on which water plants are present at the pond. As the water plant presence my change between seasons and/or due to natural succession (Begon et al. 1998), this might also help explain the cycles for the female morphs found in *Ischnura elgeans* by Svensson et al. (2005). Again more close behavioural examinations or experiments with different substrates to choose and even more detailed egg measurements might give better insight into this subject. So far we cannot exclude different reproductive strategies for the maintenance of colour morphs in coenagrionid damselflies completely. And the preference for different

oviposition substrates paired with different flight abilities might be part of reproductive strategies in the wider sense.

Acknowledgements

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Refrences

Abbott, J. K. & Svensson, E. I. 2005. Phenotypic and genetic variation in emergence and development time of a trimorphic damselfly. *Journal of Evolutionary Biology*.

Andres, J. A. & Cordero, A. 1999. The inheritance of female colour morphs in the damselfly *Ceriagrion tenellum* (Odonata, Coenagrionidae). *Heredity*, **82,** 328-335.

Andres, J. A., Sanchez-Guillen, R. A. & Cordero Rivera, A. 2000. Molecular evidence for selection on female color polymorphism in the damselfly *Ischnura graellsii*. *Evolution*, **54,** 2156-2161.

Askew, R. R. 2004. *The dragonflies of europe*. Colchester: Harley Books.

Banks, M. J. & Thompson, D. J. 1985. Lifetime mating success in the damselfly *Coenagrion puella*. *Animal Behaviour*, **33,** 1175-1183.

Begon, M., Harper, J. & Townsend, C. 1998. *Oekologie*. Heidelberg: Spektrum Akademischer Verlag.

Benke, A. C. 1970. A method for comparing individual growth rates of aquatic insects with special references to the Odonata. *Ecology*, **51,** 328-331.

Bradley, J. T. & Estridge, B. H. 1997. Vitellogenin uptake and vitellin localization in insect follicles examined using monoclonal antibodies and confocal scanning microscopy. *Invertebrate Reproduction and Development*, **32,** 245-257.

Corbet, P. S. 1999. *Dragonflies - behaviour and ecology of Odonata*. Colchester: Harley Books.

Cordero, A. 1989. Reproductive behaviour of Ischnura graellsii (Rambur) (Zygoptera: Coenagrionidae). *Odonatologica*, **18,** 237-244.

Cordero, A. 1990. The inheritance of female polymorphism in the damselfly *Ischnura graellsii* (Rambur) (Odonata: Coenagrionidae). *Heredity*, **64,** 341-346.

Forbes, M. 1994. Test of hypotheses for female-limited polymorphism in the damselfly, *Enallagma boreale* Selys. *Animal Behaviour*, **47,** 724-726.

Gorb, S. N. 1998. Visual cues in mate recognition ba males of the damselfly, *Coenagrion puella* (L.) (Odonata: Coenagrionidae). *Journal of Insect Behaviour*, **11,** 73-92.

Hinnekint, B., O, N. 1987. Population dynamics of Ischnura E. elegans (Vander Linden) (Insecta:Odonata) with special reference to morphological colour change, female polymorphism, multiannual cicles and their influence on behaviour. *Hydrobiologia*, **146,** 3-31.

Johnson, C. 1964. The inheritance of female dimorphism in the damselfly, *Ischnura damula*. *Genetics*, **49,** 513-519.

Johnson, C. 1966. Genetics of female dimorphism in *Ischnura demorsa*. *Heredity*, **21,** 453-459.

Marden, J. 1987. Maximum lift production during takeoff in flying animals. *journal of Experimental Biology*, **130,** 235-258.

Marden, J. H. 1989. Bodybuilding Dragonflies: Costs and benefits of maximizing Flight Muscle. *Physiological Zoology*, **62,** 505-521.

Nicolaro, M. L. & Bradley, J. T. 1980. Yolk proteines in developing follicles of the house cricket, *Acheta domesticus* (L.). *Journal of experimental Biology*, **212,** 225-232.

Robertson, H. M. 1985. Female dimorphism and mating behaviour in a damselfly, Ischnuar ramburi: females mimicking males. *Animal Behaviour*, **33,** 805-809.

Rolff, J. & Joop, G. 2002. Estimating condition: pitfalls of using weight as a fitness correlate. *Evolutionary Ecology Research*, **4,** 931-935.

Rüppell, G. 1989. Kinematic analysis of symmetrical flight manoeuvres of Odonata. *Journal of experimental Biology*, **144,** 13-42.

Rüppell, G., Hilfert-Rüpell, D., Rehfeldt, G. & Schütte, C. 2005. *Die Prachtlibellen Europas*. Magdeburg: Westarp Wissenschaften.

Sanchez-Guillen, R. A., Van Gossum, H. & Cordero Rivera, A. 2005. Hybridization and the inheritance of female colour polymorphism in two ischnurid damselflies (Odonata: Coenagrionidae). *Biological Journal of the Linnean Society*, **85,** 471-481.

Schenk, K., Suhling, F. & Martens, A. 2004. Egg distribution, mate-guarding intensity and offspring characteristics in dragonflies (Odonata). *Animal Behaviour*, **68,** 599-606.

Sherratt, T. N. 2001. The evolution of female-limited polymorphisms in damselflies: a signal detection model. *Ecology letters*, **4,** 22-29.

Sirot, L. K. & Brockmann, H. J. 2001. Costs of sexual interactions to females in Rammbur's forktail damselfly, *Ischnura ramburi* (Zygoteran: Coenagrionidae). *Animal Behaviour*, **61,** 415-424.

Sternberg, K. 1999. Coenagrion puella. In: *Die Libellen Badenwürttembergs, Kleinlibellen (Zygoptera)* (Ed. by Sternberg, K. & Buchwald, L.), pp. 278-287. Stuttgart: Verlag Eugen Ulmer.

Svensson, E., Sinervo, B. & Comendant, T. 2001. Density-dependent competition and selection on immune function in genetic lizard morphs. *Proceedings of the National Academy of Science*, **98,** 12561-12565.

Svensson, E. I., Abbott, J. & Härdling, R. 2005. Female polymorphism, frequency dependence, and rapid evolutionary dynamics in naural populations. *The American Naturalist*, **165,** 000-000.

Takesue, S., Onitake, K., Keino, H. & Takesue, Y. 1983. Immunocytochemical location of vitellin in the egg of the silkworm, *Bombyx mori*, during early developmental stages. *Wilhelm Roux's Archive for Developmental Biology*, **192,** 113-119.

Thompson, D. J. 1989. Lifetime reproductive success in andromorph females of the damselfly *Coenagrion puella* (L.) (Zygoptera: Coenagrionidae). *Odonatologica*, **18,** 209-213.

Tsubaki, Y., Hooper, R. E. & Siva-Jothy, M. T. 1997. Differences in adult and reproductive lifespan in two male forms of *Mnais pruniosa costalis* Selys (Odonata: Calopterygidae). *Researches on Population Ecology*, **39,** 149-155.

Wigglesworth, V. 1959. *Physiologie der Insekten*. Basel, Stuttgard: Birkhaeuser Verlag.

Wootton, R. J. 1992. Functional morphology of insect wings. *Annual Reviews in Entomology*, **37,** 113-140.

Zeiss, C., Martens, A. & Rolff, J. 1999. Male mate guarding increases females' predation risk? A case study on tandem oviposition in the damselfly Coenagrion puella (Insect: Odonata). *Canadian Journal of Zoology*, **77,** 1013-1016.

Chapter V

‘…illustrate the **danger of generalizing** in this complex field in which the phenotype may be influenced by a variety of environmental factors acting at different times in the life cycle.’

(Corbet 1999)

Female colour polymorphism in coenagrionid damselflies: a phylogenetic approach

Abstract

Within the Coenagrionidae (Odonata: Zygoptera) females of some species, of different genera show a colour polymorphism, while females of other species do not or only rudimentary. Here, we take a phylogenetic approach to ask, whether the female polymorhism arose once or several times within the Coenagrionidae. We hypothesize two different types of polymorphism (*Ischnura*-type and *Coenagrion*-type, depending on gender differences in black patterning within these types) and two possible routs for the evolution of these. To solve the question of the evolution of coenagrionid female colour polymorphism we build a molecular phylogenetic tree based on the mitochondrial gene CO II. This new phylogeny supports one of our hypothesized routs but so far we have to reject the idea of two different types of polymorphism. It seems that even in the most ancestral groups the species differed in the type of black patterning and therefore we have to assume that these differences are an ancestral trait.

Introduction

Colour polymorphisms are widespread within several species. In some cases one morph of the polymorphic species mimics an other species, usually for defense reasons. A special case of polymorphism is the intra-specific mimicry, where one sex is polymorphic and one of the morphs resembles the other sex (Andolfatto et al. 2003). This is supposed to be true for coenagrionid damselflies. In most genera of this group with several species each, females show polymorphism and one female morph is more similar to the male than the other female morph(s). Polymorphism here refers mainly to colouration but also to similarity in black patterning (Johnson 1964). This has been widely discussed and investigated over the last 20 years (e.g. Robertson 1985, Hinnekint 1987, Miller and Fincke 1999, Cordero 1992, Andres et al. 2002, Svensson et al. 2005). Robertson (1985) argued that the male mimics avoid time-consuming supernumerary matings and this should be balanced by the costs of relative greater predation on these more conspicuous females, that is more conspicuous to the human eye. Hinnekint (1987) argued that under high population densities the male mimics avoid unnecessary matings (as female damselflies can fertilize all their eggs after one mating (Corbet 1999)) while under low population densities they have a grater risk of not mating at all. Even though Fincke (2004) tries to move away from this hypothesis with her “learned mate recognition”, the idea of females mimicking males is still reminiscent.

For five species it has been shown, that the female colour polymorphism is genetically determined (Johnson 1964, Johnson 1966, Cordero 1990, Andres and Cordero 1999, Sanchez-Guillen et al. 2005) and therefore assumed to occure in all coenagrionids. Unfortunately the genetics for the inheritance have all been done in two genera of the coenagrionids (Odonata: Zygoptera, Coenagrionidae) only, in

	***Ischnura*-type** (*Ischnura elegans*)	***Coenagrion*-type** (*Coenagrion puella*)
male		
‚male-mimic‘		
typical		
some other species of that polymorphism -type	*Ischnura pumilio* *Ceriagrion tenellum* *Nehalennia speciosa*	*Coenagrion hastulatum* *Coenagrion lunulatum* *Coenagrion mercuriale* *Coenagrion ornatum* *Coenagrion pulchellum* *Enallagma cyathigerum*

Figure 1: Examples for the two different types of female colour polymorphism (*Ischnura*-type and *Coenagrion*-type) within the Coenagrionidae.

Ischnura and *Ceriagrion*. In *I. graellsii and I. elegans* green is the recessive colour (Cordero 1990, Sanchez-Guillen 2005) while in the other three species it is blue (Johnson 1964, Johnson 1966, Andres and Cordero 1999). Further more all the original work for the male mimic hypothesis has been done in *Ischnura* and only been later transfered to other coenagrionid genera as *Coenagrion* or *Enallagma* (see

summarizing table in chapter I of this thesis). In some genera it seems, that if there was anything like male mimic in some species females resemble the male on a better level than in other species (figure 1): In *Ischnura* the blue 'male-like' female not only resembles the male in colour but also in black patterning (here defined as *Ischnura*-type). Contrasting, in *Coenagrion* it has been shown that the females differ obviously in black patterning from males (*Coenagrion*-type). Furthermore the blue of the 'male-like' female *C. puella* is different from the blue in males themselves (Joop et al. under review). Therefore within the coenagrionid damselflies we might have two different types of female polymorphism, one which results in a perfect male mimic (as in *Ischnura*) and one which results in an imperfect male mimic (as in *Coenagrion*) – if it is male mimic at all.

However, so far it is not known which colour and/or black patterning is the ancestral one. We cannot argue with Haldane's sieve (stating that the recessive colour is the ancestral one) (Clarke et al. 1985), as the recessive colour is not the same for the species analyzed. This indicates different ancestors and that this polymorphism evolved at least twice within the Coenagrionidae. Similar questions about the maintenance of e.g. seasonal polyphenism and differences in patterning between sister species have been answered using phylogenetic approaches (e.g. Nylin et al. 2001, Fric et al. 2004). Here, we construct a tree of the coenagrionid damselflies using molecular markers. Subsequently we map the colour polymorphism described in the literature on the tree in order to infer the timing of the evolution of the different types of colour polymorphism. *A priori* we see two generally different pathways for the evolution of female polymorphism within the Coenagrionidae, depending on which colour and patterning are ancestral, as illustrated in figure 2.

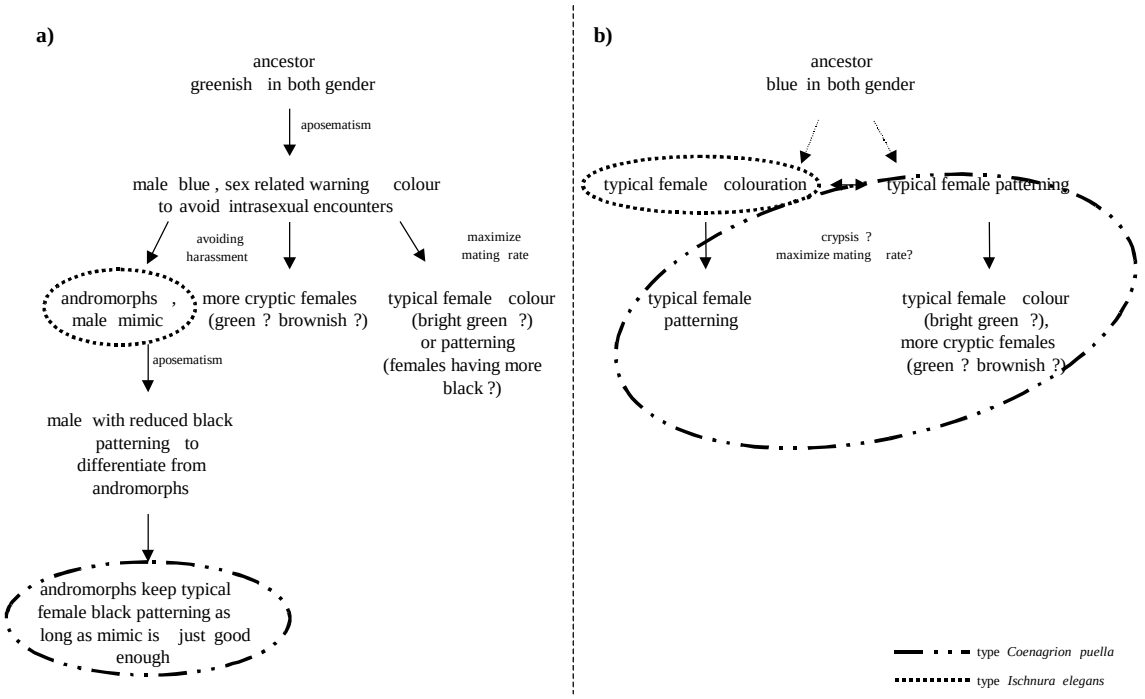

Figure 2: a) and b) present our hypothesis for the evolution of colour polymorphism in female coenagrionidae, depending on the ancestors colour. If b) was true many more species should show colour polymorphism of the *Coenagrion puella*-type than of the *Ischnura elegans*-type. (Partly based on Sherratt 2001, Sherratt and Forbes 2001, Sherratt 2002)

Methods

To obtain a molecular phylogeny of coenagrionid damselflies we used data available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). For 14 coenagrionid genera sequences were available; unfortunately not always on the same gene. For most species 18s DNA and/or the mitochondrial CO II were sequenced, therefore we build two different preliminary trees, one based on 18s and the other on CO. COII yielded a much better resolved tree (gene-convergence achieved < $4x10^{6)}$. As COII was not presented for all genera we were interested in, we decided to extract and sequence DNA of additional species (*Ischnura elegans, I. pumilio, Coenagrion johanssoni, C. mercuriale, C. pulchellum*). Specimen were preserved in pure EtOH and DNA was extracted using a 5% Chelex solution (Haine 2005). We used the

Table 1: PCR mastermix

substance	µl
dd H2O	3.95
pcr buffer	1.00
MgCl2	1.00
dNTPs	1.00
forward primer S2837	0.50
reverse primer A3571	0.50
taq	0.05
DNA	2.00

primers from Brown et al. (2000): S2837 (5'-GGTAGATCAATYTCIATRATAGG-3') and A3571 (5'-GTAACTAGAATACGYACTTGIGCTTG-3'), following a standard PCR protocol (mastermix and thermal cycling program see table 1 and 2). Samples were run on a 2% agarose gel for 65 min at 150V. As we only got one band per sample, these were cleaned using the kit ExoSAP-IT (USB Corporation) and Lark Technologies Inc. did the sequencing, using Applied Biosystems BigDye Terminator Mix version 1.1. Sequence chromatograms were edited using SeqScape v2.1 (Applied Biosystems) and aligned using Clustal X (Thompson et al. 1997).

Table 2: PCR thermal cycling program

Temperature [°C]	duration [min]	cycling	step
94	3	1x	initial denaturation
94	0.5	45x	denaturation
60 (to 48)	0.5	decrease for 1°C over 12 cycles, run at 48°C for another 31 cycles	annealing
72	1	45x	extension
72	5	1x	final extension
10	hold		

Data analysis

We used MrModeltest version 2.2 (Nylander 2004) to identify the optimal substitution model, based on likelihood values for the substitution models that are implemented in MrBayes version 3.1 (Hulsenbeck and Ronquist 2001) and generated in PAUP* 4.10b (Swofford 2000). Phylogenetic inference was conducted using MrBayes version 3.1 (Hulsenbeck and Ronquist 2001) using a general time-reversible model (GTR) with invariant sites (I) and a gamma distribution (G) (GTR+I+G). Two independent analyses were run for the Markov chain Monte Carlo (MCMC)

simulation, each with four chains (three heated, one cold), starting from a random topology. A total of 3000000 generations were simulated for each chain, with tree sampling every 100 generations and a total of 30000 trees. The first 25% of these trees (7500 generations) were discarded as the burnin. The remaining 22500 trees were used to build a 50% majority rule consensus tree with *Orthetrum triangulare* as outgroup (figure 3).

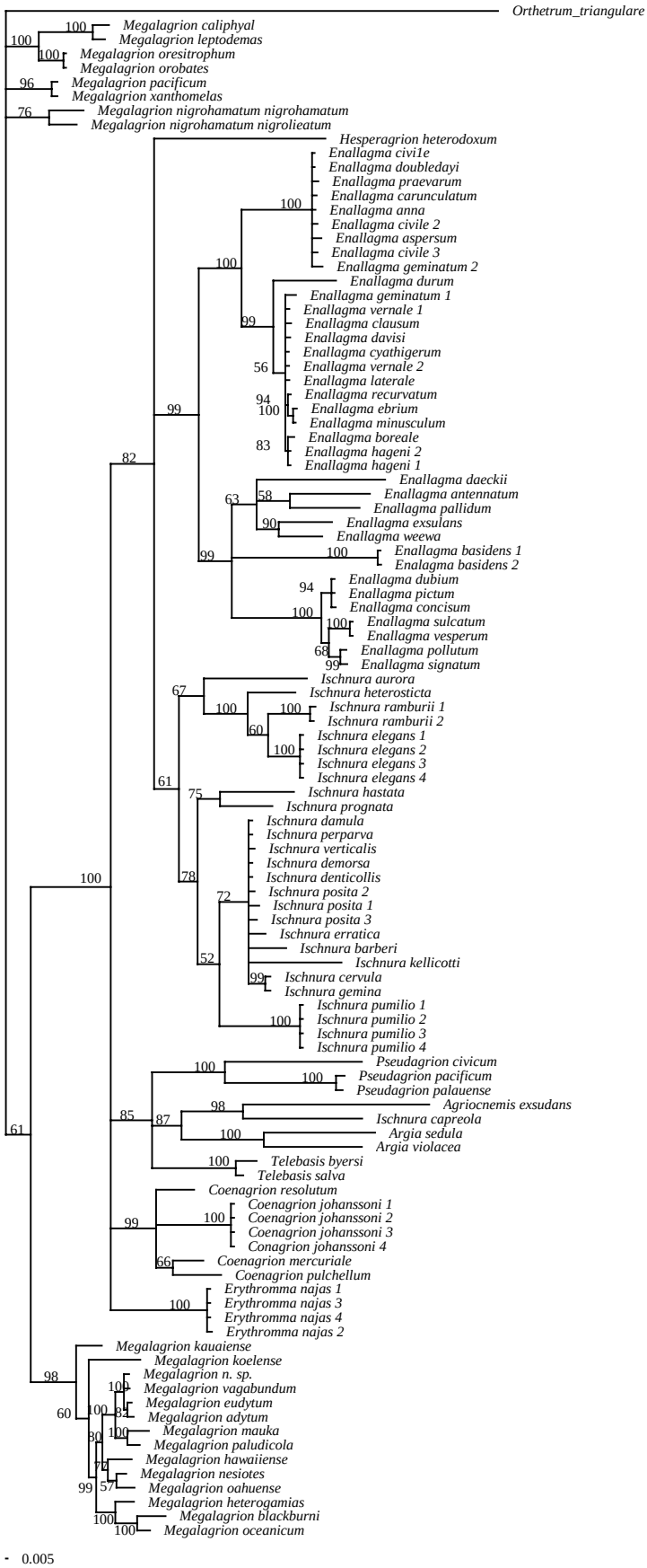

Figure 3: Molecular Coenagrionidae phylogeny, based on COII. Numbers above branches give Bayesian support, branch length are given in real proportion. *Orthetrum triangulare* was used as an outgroup.

Results and Discussion

The resulting tree (figure 3) is supported by the findings of other molecular phylogenies, which include some coenagrionid species (Hovmöller et al. 2002, Saux et al. 2003, Kjer 2004). The morphology based tree by O'Grady and May (2003) also supports, that Enallagma and Coenagrion cluster parallel to each other, but sees Ischnura as a more ancestral group. Unfortunately bootstrap supports in this tree are not very high for the Coenagrionidae.

On the base of the resulting tree (figure 3) we find the genus *Megalagrion*, which is endemic on Hawaii. In this genus there are species with red as well as with blue males and with green females (Polthemus and Asquith 1996). As the resolution on this node is low (61) we assume that all *Megalagrion* group together. On the next node the tree branches in four bigger groups (figure 3): (i) *Enallagma* + *Ischnura*; (ii) *Pseudagrion* + *Agriocnemis* + *Ischnura capreola* + *Argia*; (iii) *Coenagrion*; and (iv) *Erythromma*. A simplified version of the tree is given in figure 4, here we can see how the different polymorphism-types cluster. The resolution of the presented groups (i) – (iv) is well supported. The separation of *Enallagma* and *Ischnura* in group (i) is not as well supported, but probably several *Ischnura* groups cluster parallel to one *Enallagma* group, as was found in other molecular phylogenies (Hovmöller et al. 2002, Saux et al. 2003, Kjer 2004). The species in group (ii) represent the *Ischnura*-type (Dunkle 1990, Westfall and May 1996, Tarboton and Tarboton 2005) while group (iii) presents the *Coenagrion-type* (Sternberg 1999). *Erythromma* in group (iv) has no colour polymorphism, in black patterning it is close to the *Ischnura*-type though (Askew 2004).

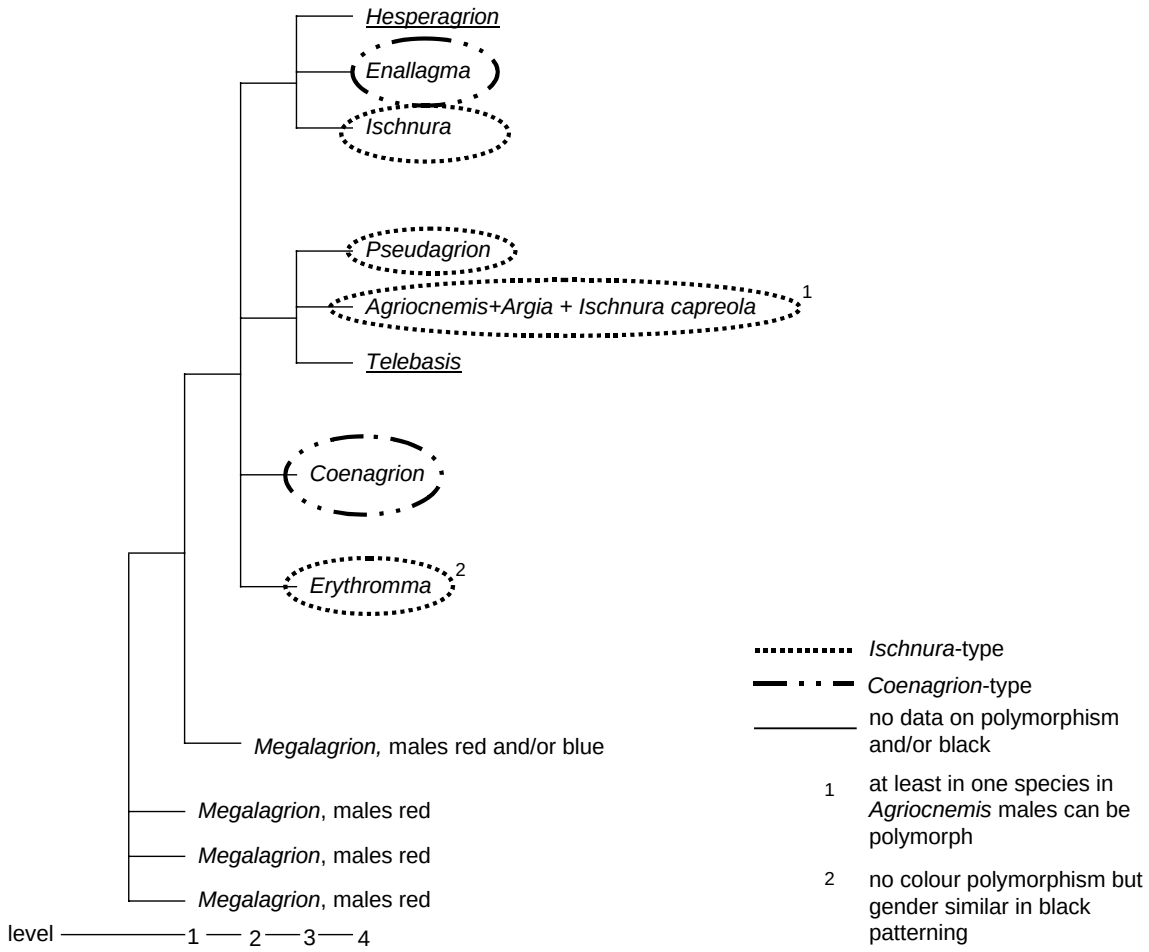

Figure 4: Simplyfied phylogeny, based on figure 3. It is shown which groups belong to which type of colour polymorphism, the *Ischnura*- or the *Coenagrion*-type.

There seems to be no clear pattern for black patterning within the basal genus *Megalagrion*. The sexes can be similar in some species, varying from not much black to completely black between species, while females have a higher black content than males in other species. Therefore both types of black patterning exist parallel at the base of the tree presented and we cannot define the ancestral type.

For male colouration, the ancestral might not be blue or green but red, as in the basal groups of *Megalagrion* males are red. Only on the next higher level we find red or blue males and in the species *M. hawaiiense* males are even colour polymorph and can be either red or blue. Some other species, as *Ceriagrion tenellum* or *Pyrrhosoma nymphula* which could not be included in this phylogeny have red males as well. Based on the findings by Hovmöller et al. (2002) with a combined 18S + 28S

tree we assume *Pyrrhosoma* to cluster on a higher level, eventually between Megalagrion (level 2) and level 3 in figure 2. These species follow the *Coenagrion*-type.

Females however are green or greenish even in the basal *Megalagrion* group. In the higher *Megalagrion* group some species have not green but brownish females but are not polymorph in colour. This might be a first hint within this coenagrionid tree of selection acting on female colour. On the third level of the tree we find E*rythromma*, here females are brownish and males blue. But we also find *Coenagrion*, where females are polymorph, blue or green while males are always blue. Therefore *Coenagrion* is the first group in which female colour polymorphism evolved. To the best of our knowledge in all genera of the next, the fourth level have colour polymorphic females.

According to this we did not find support for the pathway proposed by Sherratt (2001), Sherratt and Forbes (2001), Sherratt (2002) and our own conclusions, as illustrated in figure 2. Pathway a) is close to our findings though, if we include red as an alternative male sex-related warning colour. In a next step female polymorphism might have evolved for the reasons discussed above. As no species with red males and females could be included on the higher level of the presented phylogeny we can only speculate, if in this species the other female morphs were lost secondary and only the male-like colour got maintained. The yellow-greenish antehumeral stripes in some females of *P. nymphula* might be a hint for this. The hypothesized step that could explain the evolution of the *Coenagrion*-type out of the *Ischnura*-type probably has to be rejected. So far we cannot differentiate, whether the two presented options for black patterning are an ancestral trait or if one form was lost at one point and later on evolved again, in the latter case maybe for the assumed reasons of sex related warning.

Several other morphological and molecular trees exist which include coenagrionid species (e.g. Hovmöller et al. 2002, O'Grady and May 2003, Rehn 2003,Saux et al. 2003, Kjer 2004) or focus on one genus of the Coenagrionidae (Brown et al. 2000, Turgeon and McPeek 2002, Turgeon et al. 2005). Non of these includes all coenagrionid species or even genera. However, comparing those trees with the one presented above, we finde support for our data for those genera included. To obtain more precise answers on the evolution of colour polymorphism however, it seems reasonable to build a super-tree as a next step, including as many data on morphology and genetics as possible.

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References

Andolfatto, P., Scriber, J. M. & Charlesworth, B. 2003. No association between mitochindrial DNA haplotypes and a female-limited mimicry phenotype in *Papilio glaucus*. *Evolution*, **57,** 305-316.

Andres, J. A. & Cordero, A. 1999. The inheritance of female colour morphs in the damselfly *Ceriagrion tenellum* (Odonata, Coenagrionidae). *Heredity*, **82,** 328-335.

Andres, J. A., Sanchez-Guillen, R. S. & Cordero Rivera, A. 2002. Evolution of female colour polymorphism in damselflies: testing the hypothesis. *Animal Behaviour*, **63,** 677-685.

Askew, R. R. 2004. *The dragonflies of europe*. Colchester: Harley Books.

Brown, J. M., McPeek, M. A. & May, M. L. 2000. A phylogenetic perspective on habitat shifts and diversity in the North American *Enallagma* damselflies. *Molecular Ecology*, **11,** 1989-2001.

Clarke, C., Clarke F. M. M., Collins, S. C., Gill, A. C. L. & Turner, J. R. G 1985. Male-like females, mimicry and transvestism in butterflies (Lepidoptera: Papilionidae). *Systematic Entomology*, 10, 257-283.

Corbet, P. S. 1999. *Dragonflies - behaviour and ecology of Odonata*. Colchester: Harley Books.

Cordero, A. 1990. The inheritance of female polymorphism in the damselfly *Ischnura graellsii* (Rambur) (Odonata: Coenagrionidae). *Heredity*, **64,** 341-346.

Cordero, A. 1992. Density-dependent mating success and colour polymorphism in females of the damselfly *Ischnura graellsii* (Odonata: Coenagrionidae). *Journal of Animal Ecology*, **61,** 769-780.

Dunkle, S. W. 1990. *Damselflies of Florida, Bermuda and the Bahamas*. Gainsville: Scientific Publishers.

Fincke, O. M. 2004. Polymorphic signals of harassed odonates and the males that learn them support a novel frequency-dependent model. *Animal Behaviour*, **67,** 833-845.

Fric, Z., Konvicka, M. & Zrzavy, J. 2004. Red & black or black & white? Phylogeny of the *Araschnia* butterflies (Lepidoptera: Nymphalidae) and evolution of seasonal polyphenism. *Journal of Evolutionary Biology*, **17,** 265-278.

Haine, E. R. & Cook, J. M. 2005. Convergent incidents of Wolbachia infection in fig wasp communities from two continents. *Proceedings of the Royal Society of London, B*, **272,** 421-429.

Hinnekint, B., O, N. 1987. Population dynamics of Ischnura E. elegans (Vander Linden) (Insecta:Odonata) with special reference to morphological colour change, female polymorphism, multiannual cicles and their influence on behaviour. *Hydrobiologia*, **146,** 3-31.

Hovmöller, R., Pape, T. & Källersjö, M. 2002. The Palaeoptera Problem: Basal Pterygote Phylogeny Inferred from 18S and 28S rDNA Sequences. *Cladistics*, **18**, 313-323.

Hulsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17,** 754-755.

Johnson, C. 1964. The inheritance of female dimorphism in the damselfly, *Ischnura damula*. *Genetics*, **49,** 513-519.

Johnson, C. 1966. Genetics of female dimorphism in *Ischnura demorsa. Heredity*, **21**, 453-459.

Kjer, K. M. 2004. Aligned 18S and Insect Phylogeny. *Systematic Biology*, **53**, 506-514.

MacDougall-Shackleton, E. A. & MacDougall-Shackleton, S. A. 2001. Cultural and genetic evolution in mountain white-crowned sparrows: Song dialects are associated with population structure. *Evolution*, **55,** 2568-2575.

Majerus, M. E. N. 1989. Melanic polymorphism in the peppered moth, Biston-Betularia, and other Lepidoptera. *Journal of Biological Education*, **23,** 267-284.

Mikolajewski, D. J. & Johansson, F. 2002. Morphological and behavioral defenses in dragonfly larvae: trade compensation and co-specialization. *Behavioral Ecology*, **15**, 614-620.

Miller, M. N. & Fincke, O. M. 1999. Cues for mate recognition and the effect of prior experiences on mate recognition in *Enallagma* damselflies. *Journal of Insect Behaviour*, **12,** 801-814.

Nylander, J. A. A. 2004. MrModeltest 2.2. Uppsala: distributed by the author.

Nylin, S., Nyblom, K., Ronquist, F., Janz, N., Belicek, J. & Källersjö, M. 2001. Phylogeny of *Polygona*, *Nymphalis* and related butterflies (Lepidoptera: Nymphalidae): a total-evidence analysis. *Zoological Journal of the Linnean Society*, **132,** 441-468.

O'Grady, E. W. & May, M. L. 2003. A phylogenetic reassessment of the subfamilies of Coenagrionidae (Odonata: Zygoptera). *Journal of Natural History*, **37,** 2807-2834.

Poltehmus, D. & Asquith, A. 1996. *Hawaiian Damselflies. A Field Identification Guide*. Honolulu: Bishop Museum Press.

Rehn, A. C. 2003. Phylogenetic analysis of higher-level relationships of Odonata. *Systematic Entomology*, **28,** 181-239.

Robertson, H. M. 1985. Female dimorphism and mating behaviour in a damselfly, Ischnuar ramburi: females mimicking males. *Animal Behaviour*, **33,** 805-809.

Sanchez-Guillen, R. A., Van Gossum, H. & Cordero Rivera, A. 2005. Hybridization and the inheritance of female colour polymorphism in two ischnurid damselflies (Odonata: Coenagrionidae). *Biological Journal of the Linnean Society*, **85**, 471-481.

Saux, C., Simon, C. & Spicer, G. S. 2003. Phylogeny of the Dragonfly and Damselflly Order Odonata as inferred by mitochondrial 12S ribosomal RNA Sequences. *Annales of the Entomological Society of America*, **96,** 693-699.

Sherratt, T., N & Forbes, M., R. 2001. Sexual differences in coloration of Conagrionid damselflies (Odonata): a case of intraspecific aposematism? *Animal Behaviour*, **62,** 653-660.

Sherratt, T. N. 2001. The evolution of female-limited polymorphisms in damselflies: a signal detection model. *Ecology letters*, **4,** 22-29.

Shiga, S., Yasuyama, K., Okamura, N. & Yamaguchi, T. 2002. Neural- and endocrine control of flight muscle degeneration in the adult cricket, *Gryllus bimaculatus*. *Journal of Insect Physiology*, **48,** 15-24.

Sternberg, K. 1999. Coenagrion puella. In: *Die Libellen Baden Württembergs, Kleinlibellen (Zygoptera)* (Ed. by Sternberg, K. & Buchwald, L.), pp. 278-287. Stuttgart: Verlag Eugen Ulmer.

Swofford, D. L. 2000. PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Massachusetts: Sinauer Associates.

Tarboton, W. & Tarboton, M. 2005. *A fieldguide to the Damselflies of South Africa*. privately published.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25,** 4876-4882.

Turgeon, J. & McPeek, M. A. 2002. Phylogeographic analysis of a recent radiation of *Enallagma* damselflies (Odonata: Coenagrionidae). *Molecular Ecology*, **11,** 1989-2001.

Turgeon, J., Stoks, R., Thum, R. A., Brown, J. M. & McPeek, M. A. 2005. Simultaneous uaternary radiations of three damselfly clades across the Holarctic. *The American Naturalist*, **165**.

Westfall, M. J. & May, M. L. 1996. *Damselflies of North America*. Gainsville: Scientific Publishers.

‘ [Natural selection as] any consistent difference in fitness among phenotypically different biological entities.’

(Futuyma 1990)

‘... from the perspective of evolutionary biology, sexual selection is simply a subset of natural selection.’

(Fairbairn and Reeve 2001)

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Gerrit Frauke Joop

Curriculum vitae

Geburtsdatum	13. April 1977
Geburtsort	Braunschweig
Nationalität	deutsch
Privatadresse	Wabestr. 33
	38106 Braunschweig
Telefon	0531-3907839
e-mail	g.joop@tu-bs.de

Bildungsgang

1983-1987	Grundschule Meine
1987-1989	Orientierungsstufe Meine
1989-1996	Otto-Hahn-Gymnasium Gifhorn
Oktober 1996	Aufnahme des Studiums zur Diplom Biologin an der Technischen Universität Braunschweig
September 1998	Biologie-Vordiplom an der Technischen Universität Braunschweig
April 2001	Diplomarbeit an der Technischen Universität Braunschweig
bis Februar 2002	in Kooperation mit der University of Sheffield, UK
April 2002	Diplom der Biologie, Gesamtnote „sehr gut“
Juni bis Oktober 2002	Forschungsprojekt an der University of Reading, UK
seit November 2002	Dissertationsprojekt an der Technischen Universität Braunschweig, Zoologisches Institut, Prof. Dr. Rüppell in Zusammenarbeit mit der University of Sheffield, Department of Animal and Plant Sciences, Dr. Rolff

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Rolff, J. und Joop, G. 2002. Estimating condition: pitfalls of using weight as a fitness surrogate, *Evolutionary Ecology Research* 4, 931-935.

Mikolajewski, D. J., Brodin, T., Johansson, F. und Joop, G 2005. Phenotypic plasticity in gender specific life-history: effects of food availability and predation, *Oikos* 110, 91-100

Schütte, C., Joop, G., Mikolajewski, D. J., Mosch, E. C., Schenk, K. und Wohlfahrt, B. 2005. Die FFH-Art *Coenagrion mercuriale* (CHARPENTIER 1840) im Niedermoorgebiet „Großes Bruch“ in Niedersachsen (Odonata: Coenagrionidae), *Braunschweiger Naturkundliche Schriften*, in press.

Wohlfahrt, B., Mikolajewski, D. J., Joop, G. und Suhling, F. 2005. Avoiding fish predators – does species matter? An approach in larval odonates, *Freshwater Biology*, in press.

Tagungsbeiträge und Seminare allgemeiner Art

Joop, G. 2001. Narural enemies alter investment in condition and immunity in larval damselflies (Coenagrion puella, Odonata) (Poster), *European Science Foundation meeting “Ecological immunity in Arthropods”*, Castleton, UK.

Joop, G. 2002. Gestresste Libellen: Auswirkungen natürlicher Feinde (Vortrag), *Gesellschaft deutschsprachiger Odonatologen*, Worms, Deutschland.

Joop, G. 2003. Bildung geflügelter Blattlausmorphen, ein pleiotroper Effekt? (eingeladenes Seminar), Universität Würzburg, AG Prof. Dr. Poethke.

Joop, G. 2003. Wing induction in aphids due to predation and crowding: Is it a pleiotropic effect? (Poster), *Royal Entomological Society*, Reading, UK.

Joop, G. 2003. Wing induction in aphids due to predation and crowding: A pleiotropic effect (Poster), *European Conference on Behavioural Biology*, Groningen, Niederlande.

Mikolakewski, D.J., Küpper, O. und Joop, G. 2003. Influence of cannibalism and predation on life history traits and immunity in larval odonates (Poster), *European Conference on Behavioural Biology*, Groningen, Niederlande.

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